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THE FATE OF THE ECTODERM AND ENDODERM OF HYDRA WHEN CULTURED INDEPENDENTLY

E. J. PAPENFUSS AND N. A. H. BOKENHAM[1]

(*From the Zoölogical Laboratory of the University of Cape Town*)

It was recently found by Gilchrist (1937) that pieces of ectoderm of scyphozoan polyps, presumably the scyphistomas of *Aurellia*, are able to regenerate small complete polyps; while endodermal pieces are unable to regenerate although they may remain alive for several days.

It is of considerable interest that a complete polyp can be regenerated from ectoderm alone as this phenomenon necessitates the formation of the endodermal layer from a tissue composed of cells of a completely different structure. In view of this fact it becomes of importance to know whether the newly regenerated tissue layer is formed by a transformation of certain of the differentiated cells; or whether undifferentiated embryonic cells are activated to form anew the characteristic cell types of the lacking tissue, as Wilson and Penney (1930) found to be true for many cell types in regeneration from dissociated sponge tissue.

Gilchrist did not study the origin of the endodermal layer in his regenerates but suggests that it may have been formed by the interstitial cells of the ectoderm. The present study was undertaken with the object of casting light upon this question. *Hydra* is a favorable organism for studying this problem as the endoderm can readily be separated from the ectoderm in the living condition (Papenfuss, 1934, pp. 227–228). Accordingly the two cellular layers were isolated and cultured independently. Both green and brown *Hydra* (species undetermined) were employed in each series of experiments.

Attempts to Obtain Regeneration from the Ectodermal Layer

In order to obtain a *Hydra* consisting only of ectoderm, it was first necessary to cut off the head of the specimen since it was not possible to remove the endoderm from the tentacles. The specimen was next everted and the endoderm removed. The *Hydra* now consisting only

[1] The experimental phase of this study was carried out by E. J. Papenfuss while the histological part was done by N. A. H. Bokenham.

of the ectodermal layer was then carefully examined under a higher magnification to make certain that fragments of endoderm had not remained behind. Hydras with buds were not used as the endoderm could not be removed from these parts. In order to be certain that the entire endodermal layer is removed by this method, paraffin sections were made of individuals which had been so treated. Such sections show the specimens to be without endoderm. For the histological phase of the work, the specimens were fixed in formol-acetic-acid, stained in safranin or Heidenhain's haematoxylin, and fast green. The latter was found to be a good stain for the mesogloeal layer.

After the endoderm had been removed from the *Hydra* which was to be cultured, the specimen was placed in a small Stender dish containing pond water. Filtered pond water was used throughout the experiments, and all the dishes and instruments were kept chemically clean. In order to ascertain if the conditions were favorable for the cultured tissue, a normal *Hydra* was placed in each dish.

The data for this part of the paper were taken from 30 consecutive experiments. At the beginning of the study a few trial experiments were made which were not taken into consideration in the compilation of the results.

In none of the experiments in which the ectoderm alone was cultured did a *Hydra* develop. The tissue always disintegrated, while the controls lived and appeared entirely normal. In 14 of the experiments the ectodermal tissue had completely disintegrated within 24 hours, in 8 of the experiments the tissue did not disintegrate until the second day, in 7 of the experiments a small piece of tissue persisted until the third day, and in one case a minute fragment of what appeared to be ectodermal tissue persisted until the seventh day.

It may be noted that Gilchrist used only pieces of ectodermal tissue in his experiments while in the present work the entire ectodermal layer with the exception of the ectoderm of the head was used, as it seemed likely that regeneration would occur more readily from a large piece of tissue than from a small one.

Since it was necessary in these experiments to remove the head of the *Hydra* and to evert the body in order to make the endoderm accessible, a series of experiments were performed to determine whether a *Hydra* so treated can live and regenerate. The results from 35 experiments may be summarized as follows: In 30 of the experiments the *Hydra* lived and became normal; that is, the ectoderm again formed the external layer and tentacles developed. In the remaining five experiments, the *Hydra* died. In practically all the experiments in which the *Hydra* lived, the ectoderm was again on the exterior within 24 hours.

The results of these experiments thus show that the majority of hydras will live under such conditions although an occasional one may die. It may be noted that in the experiments in which the *Hydra* did not live, the specimen was badly damaged while it was being everted. In the experiments in which the isolated ectodermal layer was cultured, such specimens were discarded as it was then difficult to remove the endoderm.

From the first series of these experiments it may be concluded that the ectodermal layer of *Hydra* alone is not able to regenerate an individual, while the second series of experiments shows that the failure of the ectodermal layer to live and regenerate cannot be attributed to the treatment the *Hydra* had received prior to the removal of the endodermal layer. In order to determine whether the isolated endodermal tissue is capable of regeneration, the experiments set forth in the following section were performed.

Attempts to Obtain Regeneration from Endodermal Tissue

It was not possible to obtain a complete endodermal layer by removing the ectoderm as the latter is rigid and difficult to cut. It was accordingly necessary to evert the *Hydra* and to remove the endoderm by scraping it off with a fine glass needle. This tissue, however, dissociates very readily and comes off in small fragments. Since Peebles (1897) has shown that the smallest portion of a *Hydra* that is capable of regeneration is a sphere whose diameter is $\frac{1}{6}$ mm., it was considered necessary to fuse the fragments together so that a piece of tissue larger than this minimum size could be obtained for culturing. In the green species the fusion masses measured from 0.8 mm.–0.9 mm. in diameter and in the brown form they measured from 0.9 mm.–1.2 mm. in diameter. In a few experiments with both the green and the brown *Hydra*, the endodermal fragments from two individuals of the same species were allowed to fuse in order to obtain a larger mass of tissue.

As the endodermal tissue of the green *Hydra* dissociates more readily than that of the brown, it was necessary to employ somewhat different methods for the two forms. When working with the green *Hydra*, the fragments were transferred by means of a fine pipette to a small hole in an agar bed. They were then placed as close together as possible so as to be in a position favorable for fusion. Only a small amount of water was allowed on the agar to prevent the fragments from floating apart. The slide was examined frequently and water added when necessary to prevent the tissue from becoming dry. If the hydras were fresh from the pond, complete fusion occurred in this form in approximately 15

minutes. Hydras which had been in the laboratory for some time were unsuitable for this type of experiment unless they were from a well-established culture.

When brown hydras were used it was found that larger fragments of endoderm could be obtained as the endoderm in this form dissociates less readily than in the green *Hydra.* It was then more practicable to place each individual fragment in contact with the other fragments as soon as it was removed from the *Hydra.* This was done with a fine glass needle. As much water as possible was withdrawn to prevent the fragments from floating apart. As the process of fusion takes longer in the brown *Hydra,* the slide was kept in a damp chamber, examined frequently, and water added when necessary to prevent the tissue from becoming dry.

After the tissue had fused securely, the fusion mass was taken up in a pipette with a comparatively large opening and transferred to a small dish containing filtered spring water. A normal *Hydra,* to be used as a control, was placed in the dish with the endodermal fusion mass.

This type of experiment is difficult to perform since the endoderm easily dissociates. The writers, however, carried through to completion 12 experiments. In no case was a new *Hydra* regenerated from the endodermal tissue, although the controls lived and appeared entirely normal. In the majority of experiments the fusion mass had completely disintegrated within 24 hours while in a few cases minute fragments of the tissue could still be recognized after two or three days.

The results of these experiments show that the endoderm of *Hydra* alone is not capable of regenerating an individual. It is of interest to note that similar fusion masses composed of both ectoderm and endoderm will regenerate complete new hydras (Issajew, 1926; Papenfuss, 1932, 1934; Weimer, 1934).

Discussion and Conclusions

The object of the present study was to ascertain if a *Hydra* can be regenerated from either ectoderm or endoderm alone. The results of the experiments were entirely negative.

Since *Hydra* has great regenerative abilities, being able to form a new individual from a small fragment of tissue (composed of both ectoderm and endoderm) only $\frac{1}{6}$ mm. in diameter (Peebles, 1897), it is of considerable interest that a regenerate is not formed when practically an entire cellular layer is cultured. In attempting to explain these results, the writers suggest that a regenerate did not develop because

the differentiated cells of one tissue layer are unable to transform into the cell types characteristic of the lacking tissue layer, and the undifferentiated cells (the embryonic interstitial cells) do not become activated to regenerate this tissue.

That differentiated cells do not transform into different cell types has been shown to be true previously by Wilson and Penney (1930), who found that in reunition of sponge tissue from dissociated cells, the specialized cells (the collar cells) which are carried over into the reunition mass do not change their characteristics but become the collar cells of the new individual. Likewise in reunition in *Hydra* the specialized foot cells retain their identity and function as the foot cells of the new individual (Papenfuss, 1934).

As the unspecialized interstitial cells of *Hydra* which are present in both the ectoderm and the endoderm are known to possess certain formative powers (Kleinenberg, 1872; Schneider, 1890; McConnell, 1932; and others), it seemed possible that these cells might regenerate anew the lacking tissue layer. However, judging from our results, it may be concluded that the formative powers of the interstitial cells are limited. According to Kanajew (1930), the interstitial cells play a subordinate rôle in regeneration in *Hydra,* the regenerated part, at least as regards the endoderm, being formed at the expense of the nearby differentiated tissue. In the sponge, however, the unspecialized mesenchyme cells possess great formative powers, being able to reëstablish very many of the characteristic cell types of a regenerate (Wilson and Penney, 1930).

It should be noted that Ischikawa (1890) previously attempted to obtain *Hydra* regenerates from ectoderm alone. This author obtained ectodermal tissue by destroying the endoderm with acetic acid vapor. No regenerates were formed, however, and Ischikawa concluded that the interstitial cells were unable to give rise to the lacking tissue layer. Schulze (1918) suggests that the failure of the ectodermal tissue to regenerate was owing to the lack of nourishment normally supplied by the endoderm. Schulze's conclusion, however, loses support by the fact that isolated endodermal tissue also fails to regenerate, as was found in the present study.

The results of our experiments are at variance with those of Gilchrist (1937), who found that pieces of ectoderm of a scyphistoma are able to regenerate complete new individuals. As the disposition of the endoderm of a scyphozoan polyp is somewhat complicated by the invasion of the ectodermal cells in the four taeniolae, it is possible that Gilchrist did not succeed in removing all the endoderm from the fragments.

Acknowledgment

The writers wish to thank Professor W. A. Jolly for the loan of a dissecting microscope and for granting them the facilities of the Department of Physiology where part of the work was done. They further wish to thank Dr. M. A. Pocock for aid in the collecting of material.

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[2] The authors did not have access to the papers marked with an asterisk but have seen the abstract of Kanajew's work in *Biological Abstracts*, Vol. 5 (II), 1931, p. 2952; and have read Schulze's (1918) account of the work of Ischikawa.

THE REPRODUCTIVE SYSTEM AND SPERMATOGENESIS OF LIMACINA (SPIRATELLA) RETROVERSA (FLEM.) [1]

SIDNEY C. T. HSIAO

(From the Biological Laboratories, Harvard University and the Woods Hole Oceanographic Institution,[2] Woods Hole, Massachusetts)

INTRODUCTION

Limacina retroversa was described as early as 1823 by Fleming under the name of "*Heterofusus retroversus,*" but very little is known about its internal structure, its method of reproduction and its life history. Writing in 1924—one hundred years later—Bigelow mentioned that "' Nothing is known of the reproduction of *Limacina retroversa* in the Gulf of Maine. . . ." The embryonic development and the reproductive system of several species of Pteropoda have been described but no exact information on the species *L. retroversa* is available. Van Beneden described the structure of the reproductive system of *L. arctica* in 1839 but, as pointed out by Meisenheimer (1905), he made the mistake of calling the ovotestis the ovary, the vesicula seminalis the testis and the accessory glands the "gland prostatique." Studies of the embryonic development of pteropods have been made by Gegenbaur (1853, 1855), Krohn (1856), Müller (1857), Fol (1874, 1875) and Knipowitsch (1891). Meisenheimer (1905) described the comparative morphology of Pteropoda and compared the genus *Limacina* with other Euthecosomata. He considered *Limacina* as a general group and made no mention of any species. Meisenheimer also appraised the earlier work and observations of Cuvier, d'Orbigny, Van Beneden, Gegenbaur, Souleyet, Pelseneer, Knower, etc. Bonnevie (1916) made further observations on the reproductive organs of *Cuvierina* and recently Zarnik (1911) determined the chromosome number of several species of pteropods. Up to the present no one has studied the entire life history of any species of Pteropoda and it has been unknown whether any pteropods, like some other mollusks, change their sex. The reproductive system of *Limacina retroversa,* in particular, has not been studied. It

[1] The name "*Limacina retroversa*" has been in use until 1930, when Sherborn pointed out that Cuvier (1817) used the vernacular name "Limacine" and he restored de Blainville's "*Spiratella*" in place of Lamarck's (1819) "*Limacina.*" Thiele (1931) also used "*Spiratella.*" However, as "*Limacina*" has the advantage of being more commonly used and better known it is retained in this paper.

[2] Contribution No. 197 from the Woods Hole Oceanographic Institution.

is the purpose of this paper to describe the reproductive system and spermatogenesis of *Limacina retroversa* as a basis for a report on the reproductive history of this species to appear in a subsequent paper.

Material and Methods

The material was collected by the research vessel " Atlantis " of the Woods Hole Oceanographic Institution during a series of cruises in the Gulf of Maine between June, 1933 and September, 1934. The specimens were caught by vertical hauls made with a 1.5-meter Heligoland net drawn from a level near the bottom up to the surface and were fixed on board the ship in 2 per cent formalin in sea water.

Each specimen used for making permanent mounts was decalcified in 1 per cent acetic acid for a few minutes. In some cases the acid in the commercial formalin used in fixation had decalcified the specimens. The decalcified animal was then stained in toto for about one minute in a 0.1 per cent aqueous solution of Erhlich's methylene blue chloride to aid in orienting it in the paraffin block for sectioning. The stained specimen was dehydrated in two grades of 1–4 dioxene in a calcium tower and directly imbedded in 53°–55° C. paraffin. Each individual was cut in serial longitudinal sections five micra in thickness which were mounted in order on slides. The vital stain was removed when the sections were hydrated after the removal of paraffin with xylene. Standard methods of staining with Heidenhain's iron haematoxylin and orange G were used in the final staining process. Stained sections were cleared in xylene and mounted in Canadian balsam.

In order to reconstruct the different parts of the reproductive system sections from suitable individuals were drawn with a camera lucida. The tracings of every fifth or tenth section were made with a wax pencil on pieces of glass. Reconstruction of the reproductive system was made by superimposing the glass plates in order so as to determine the size and shape of each organ. These tracings were compared with Meisenheimer's plates and descriptions (1905) of similar sections of the reproductive apparatus of Euthecosomata. Meisenheimer's terminology of the different parts of the regenerative system was followed.

In the study of the spermatogenesis of *Limacina retroversa* the method for carmine smears was used in addition to the examination of permanent mounts stained in Heidenhain's haematoxylin. The specimens used for making carmine smears were collected in the summer of 1937 near Woods Hole. As soon as the animals were taken from water they were fixed for twenty minutes in a mixture of one part of acetic acid and three parts of absolute alcohol. Each *Limacina* was sepa-

rated from other planktonic material and stored in 70 per cent alcohol. It was necessary to change the preservative several times in order to get rid of the precipitate brought down by alcohol from sea water. The gonads from several individuals were dissected out under a binocular microscope and torn up into very small pieces. They were then stained with cytological aceto-carmine for a few minutes, covered with a coverglass and heated over a small flame without boiling. The slide with the material was then pressed between blotting papers to separate the cells and to remove the excessive stain. Such preparations showed the cellular structures very clearly and were used for spermatogenesis studies.

Living animals have been kept in the laboratory for a week to ten days for observation of spawning and egg-development.

Observations

The Reproductive System

The reproductive system of *Limacina retroversa* consists of a bisexual gonad, a gonoduct, accessory glands and a penis.

The Gonads.—The bisexual gonad or ovotestis constitutes the primary sexual organ. In a mature individual it is a spiral structure situated posterior to the liver and occupies the posterior or last three or four whorls of the shell with whose cavity the smooth, non-lobated coils of the ovotestis conform (Fig. 1). In specimens preserved in formalin the gonad appears, after decalcification, granular and pinkish in contrast to the yellowish-green liver with which it is continuous. In living specimens the gonads can also be distinguished from the greenish liver through the transparent shell. In sectional view, the cortical and apical regions of the mature gonads are generally occupied by the female reproductive cells and the medullary portion by the spermatic tissues. The spermatic tissues are apparently arranged in tubules in the medulla of the ovotestis, but in well-developed males they may extend to the cortex with masses of mature spermatozoa obliterating the tubular arrangement of the reproductive cells. In large females, on the other hand, the mature ova may be found in the medullary region just before they pass into the hermaphroditic duct. The proportion of masculine and feminine reproductive cells varies with the season as well as with the size of the animal.

Three different types of gonads can be distinguished. (*A*) Sexually undifferentiated gonads all of which occur in small-sized specimens; (*B*) "pure male" gonads which belong to somewhat larger animals but never to animals greater than 1.1 mm. in diameter across the largest

whorl of the shell; and (*C*) hermaphroditic gonads, or ovotestes, characteristic of all larger individuals.

A. Sexually undifferentiated gonads.—In very small specimens the gonad is sexually undifferentiated in that no definite male or female reproductive cells can be recognized. This type of gonad corresponds with the "indefinite gonads" of Coe (1931) and Orton (1926). The whole gonad is small, nearly spherical and surrounded by the liver. The pregerminative cells, all of nearly equal size, form a solid mass which is enclosed within a thin fibrous Ancel's layer consisting of elongated cells

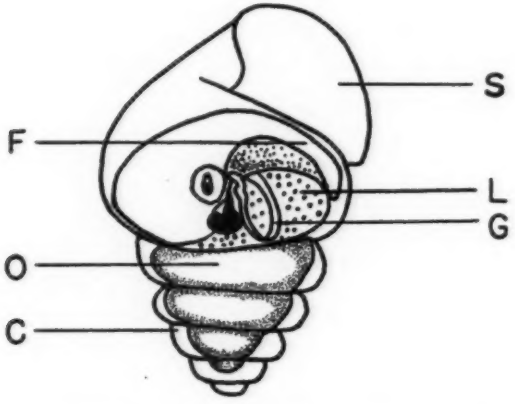

FIG. 1. Camera lucida drawing of *Limacina retroversa* (× 30), showing the gonad and the rest of the body.

C. Cavity between shell and ovotestis.
F. "Flapper" or "foot."
G. Gut.
L. Liver.
O. Ovotestis.
S. Shell opening.

with prominent nuclei. The cellular structure of the pregerminative cells is not easy to make out, for in specimens fixed with commercial formalin and stained with haematoxylin the nuclei are diffused and the structure not clear. In this, as in the other types of gonads, the fixative used tends to make the cells indistinct from each other so that they have the appearance of a syncytium. A sectional view of a young indefinite gonad is shown in Fig. 2, *A*.

B. "Pure male" gonads.—All *Limacina retroversa* larger than 0.85 mm. in diameter show sexual differentiation, for either male or female

germ cells are discernible. But nearly all, if not all, of the very small sexually differentiated individuals are protandric. Half of the protandric animals do not show any ovogenesis at all. Individuals possessing this type of gonad correspond to Coe's (1933) "true males" or Orton's (1909) "pure males." In this description they will be called "pure males." The "pure male" type of gonad is confined to small-sized *Limacina retroversa*. No individuals larger than 1.1 mm. in diameter show complete maleness in their gonadal organization. In Fig. 2, *B* a gonad without any feminine reproductive cells distinguishable from the male cells is shown. In this diagram a few clusters of spermatozoa and a few groups of spermatids are seen while many of the other cells are undergoing active spermatogenesis.

C. Hermaphroditic gonads.—Besides "pure males" sexually differentiated *Limacina* have gonads with various proportions of masculine and feminine types of tissue and show different signs of sexual activity. For convenience of description and comparison, these larger specimens, possessing ovotestes, can be divided into classes in terms of the percentage of masculine or feminine tissue in the gonad. Individuals with more than 50 per cent of the tissue of their gonads consisting of masculine reproductive cells, showing spermatogenesis, are designated as hermaphroditic males and those with less than 50 per cent of the contents of their gonads made of male reproductive cells are called hermaphroditic females. On this percentage basis the gonads can be arranged from extreme masculine to pronounced feminine types.

Gonads of individuals which are classified as hermaphroditic males are shown in Fig. 2, *C* and *D*. Figure 2, *C* shows the gonad of a mature hermaphroditic male with more than 75 per cent of its reproductive tissues consisting of masculine cells. This gonad occurred in a *Limacina* 1.2 mm. in diameter. In contrast to the "pure male" type of gonad, a peripheral layer of ovarian tissue is present, but it amounts to less than 25 per cent of the total reproductive cells in the organ. In the medullary portion clusters of mature spermatozoa are present while in the cortical region a small number of egg cells are developing. Individuals possessing this type of gonad may be of any size but most of them are comparatively small. They are smaller than the pronounced feminine type but larger than the "pure males."

Figure 2, *D* shows the ovotestis of an individual belonging to the class having one-half to three-quarters of the gonad occupied by male tissue. In this ovotestis mature cells of both sexes are seen. At a point near the beginning of gonoduct, next to the retractor muscles, in the largest whorl of the spiral organ, mature ova and spermatozoa are seen ready to leave the sex gland. This simultaneous presence of ma-

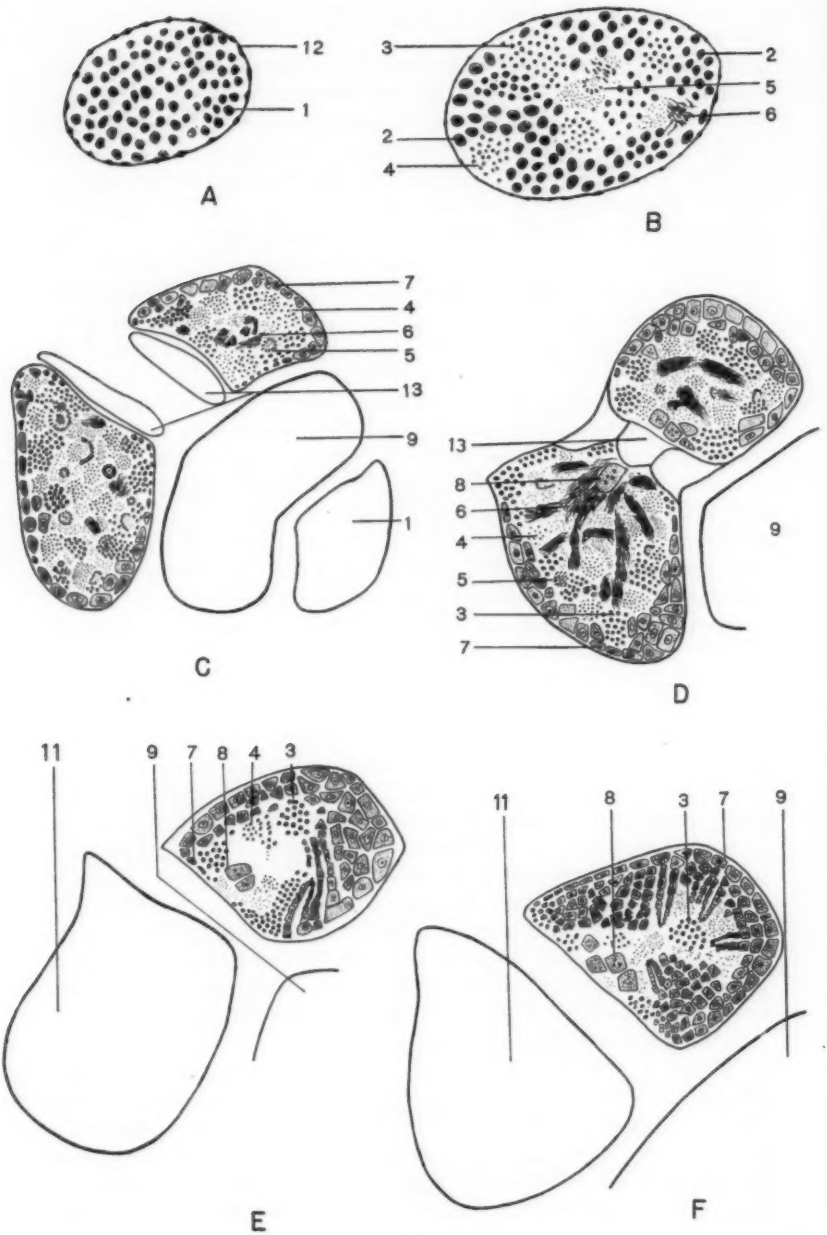

FIGURE 2

ture ova and spermatozoa near the gonoduct, or the presence of mature cells of both sexes along it, indicates that the animal is functioning both as male and female. Individuals of this type are called functional hermaphrodites, a name commonly used to designate this kind of reproductive individual among mollusks.

Animals with less than half of the gonadal tissues consisting of male reproductive cells, which are classified as hermaphroditic females, can also be divided into two groups in a similar manner: (*a*) hermaphroditic females with gonads containing 50–25 per cent of male tissue, and (*b*) hermaphroditic females with less than 25 per cent of male tissue in the gonad. These two types are represented in Fig. 2, *E* and *F*. As a general rule, gonads which are predominantly feminine are in a mature stage, containing mature ova and very often both mature ova and spermatozoa. The *Limacina* possessing these gonads are generally larger than the masculine types.

Part of a gonad with 25–50 per cent of its tissues consisting of male germ cells is shown in Fig. 2, *E*. This gonad is in a mature condition, for mature ova are found in the medullary portion of the ovotestis near the beginning of the gonoduct instead of in the cortical region where ovocytes of various stages of development are seen. Empty spaces are left in the gonad apparently by the spermatozoa which have vacated the

FIG. 2. Drawings of different types of gonads of *Limacina retroversa*.

A. Section of a complete gonad which is sexually undifferentiated.
B. A "pure male" gonad in which no ovocytes are seen—section of a complete gonad.
C. A hermaphroditic male gonad which has more than 75 per cent of its tissues made of male germ cells. Only the first or largest whorl is shown, the other two whorls being indicated by outlines.
D. First or largest whorl of a hermaphroditic male gonad with 50–75 per cent of its tissues consisting of male germ cells.
E. Right half of the first or largest whorl of a hermaphroditic female gonad which contains 25–50 per cent of male tissue.
F. Right half of the first or largest whorl of a hermaphroditic female gonad with less than 25 per cent of its tissues made of male germ cells.

1. Nucleus of pregerminative cell.
2. Nucleus of spermatogonium.
3. Primary spermatocytes.
4. Secondary spermatocytes.
5. Spermatids.
6. Spermatozoa.
7. Ovocyte.
8. Ovum.
9. Outline of second whorl of ovotestis.
10. Outline of third whorl of ovotestis.
11. Outline of left half of first whorl of ovotestis.
12. Fibrous connective tissue surrounding gonad.
13. Retractor muscles.

gonad previously. Figure 2, *F* shows a gonad which is even more predominantly feminine than that shown in Fig. 2, *E*. Less than 25 per cent of this ovotestis is made up of masculine reproductive cells.

In contrast to masculine types, the pronounced feminine hermaphro-

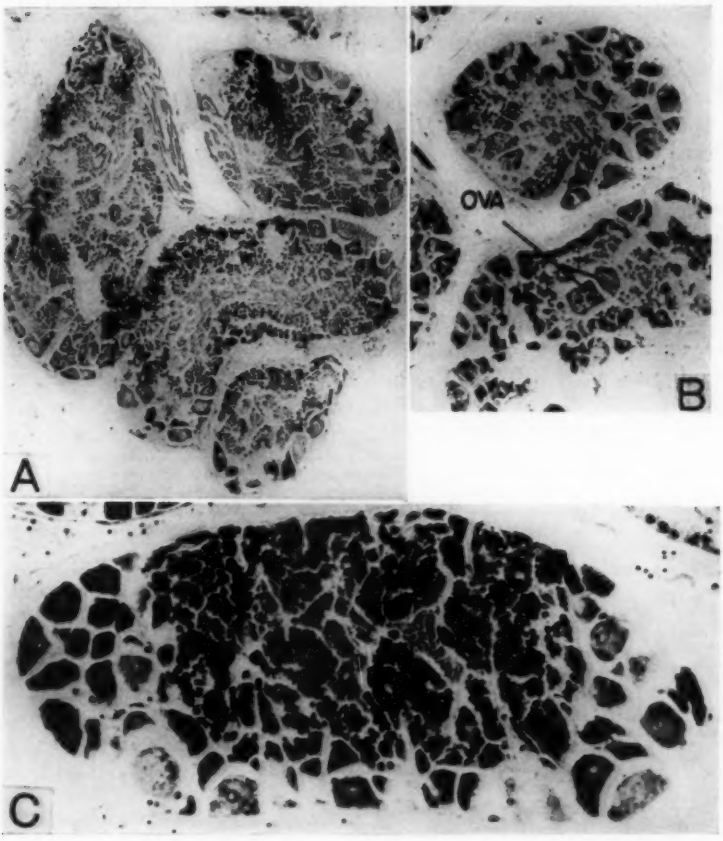

FIG. 3. Photomicrographs of sections of gonad of *Limacina retroversa*, showing hermaphroditic male and female gonads.

A. Hermaphroditic male gonad in which more than 75 per cent of the tissues consists of male reproductive cells.

B. Hermaphroditic female gonad in which 25–50 per cent of the gonadal tissues consists of male germ cells.

C. Further enlarged view of a section of the second whorl of a hermaphroditic male gonad with 50–75 per cent male tissue, showing the germ cells in greater detail.

dites possessing very little male tissue are limited to medium and, more generally, to large-sized *Limacina*. Another difference between the extremely male and female types is that while "pure males" are common, forming about one-half of the protandric males, "pure females" have never been observed. All female *Limacina retroversa* are hermaphroditic.

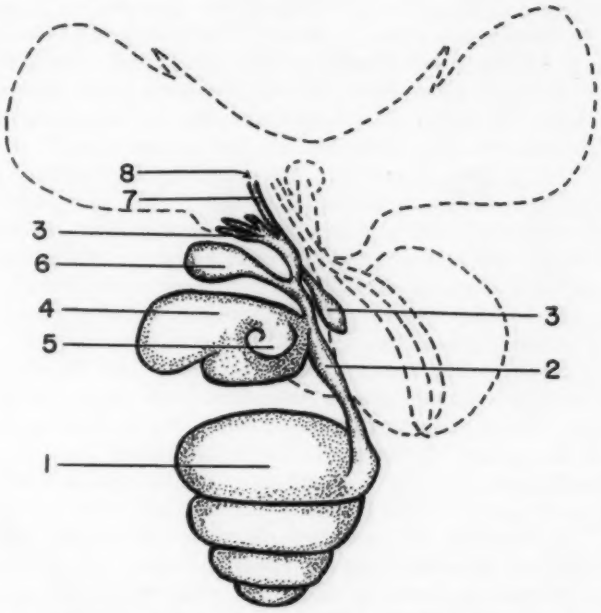

FIG. 4. Diagram of the reproductive system of *Limacina retroversa*.

1. Ovotestis.
2. Hermaphroditic duct or gonoduct.
3. Receptaculum seminis.
4. Shell gland.
5. Albumin gland.
6. Vesicula seminalis.
7. Out-going duct.
8. Genital opening.

Figure 3 shows some of the photomicrographs of sections of the gonad of *Limacina* upon which the diagrammatic drawings of Fig. 2 are based. Figure 3, *A* is a hermaphroditic male gonad with more than 75 per cent of its tissues consisting of male germ cells and Fig. 3, *B*, a hermaphroditic female gonad with 25–50 per cent of male tissue in it. Figure 3, *C* is a further enlarged view of a masculine gonad with

50–75 per cent male tissue showing the arrangement of the germ cells in greater detail.

The Accessory Organs of Reproduction.—The accessory organs of the reproductive system are diagrammatically represented in Fig. 4, which is a composite picture based upon the reconstruction from serial sections of the whole animal such as those shown in Fig. 5.

The gonoduct begins as a hermaphroditic duct for the passage of both ripe spermatozoa and ova. It opens into the ventral side of the anterior end of the spiral ovotestis and then turns to the right and passes forward, along the right side of the digestive track, to the anterior end of the body. It begins as a single tube with its wall composed of cuboidal cells, but later branches out into a pear-shaped structure (called "6" in Figs. 4 and 5). After meeting the accessory glands the gonoduct continues as the out-going duct. This part of the duct is much convoluted, leading to the external opening of the reproductive system on the right side of the mouth (8 in Fig. 4). This tube is composed of cuboidal epithelial cells with prominent nuclei and the cytoplasm of these cells contains a large quantity of secretory granules.

The accessory glands, like those of other pteropods, are very complicated structures. The largest gland, according to the terminology of Meisenheimer, is the shell gland. It is divided into three or four lobes occupying a great portion of the right side of the anterior part of the body of the animal. It opens into the hermaphroditic duct as well as the albumin gland (see Fig. 5, *D* and *E*). The shell gland is composed for the most part of large secretory cells, and tall, ciliated, columnar cells. The secretory cells are very abundant and obvious and with nuclei which are generally large and ellipsoidal, lying on the base of the cells away from the lumen of the gland, while the portion near it is filled with secretion. Perhaps the ovum stays longest in this gland on its way through the various parts of the reproductive system, for ova have been more often observed in this organ than in the others.

The albumin gland (Fig. 5, *B* to *D*) is a snail-like coiled gland associated with the shell gland but smaller than it. A diagram of its outline is shown in Fig. 4. The cells of the albumin gland are largely secretory. Their inner edge, which faces the lumen, is filled with transparent secretion when seen in sections stained with iron haematoxylin.

The vesicular seminalis (Figs. 4 and 5) is an elongated, pear-shaped organ connected with the hermaphroditic duct. It is lined with cuboidal epithelial cells. Inside this organ masses of mature spermatozoa are seen in the case of mature *Limacina*.

The receptaculum seminis is connected with the basal portion of the out-going duct. It consists of two portions. The first portion is much

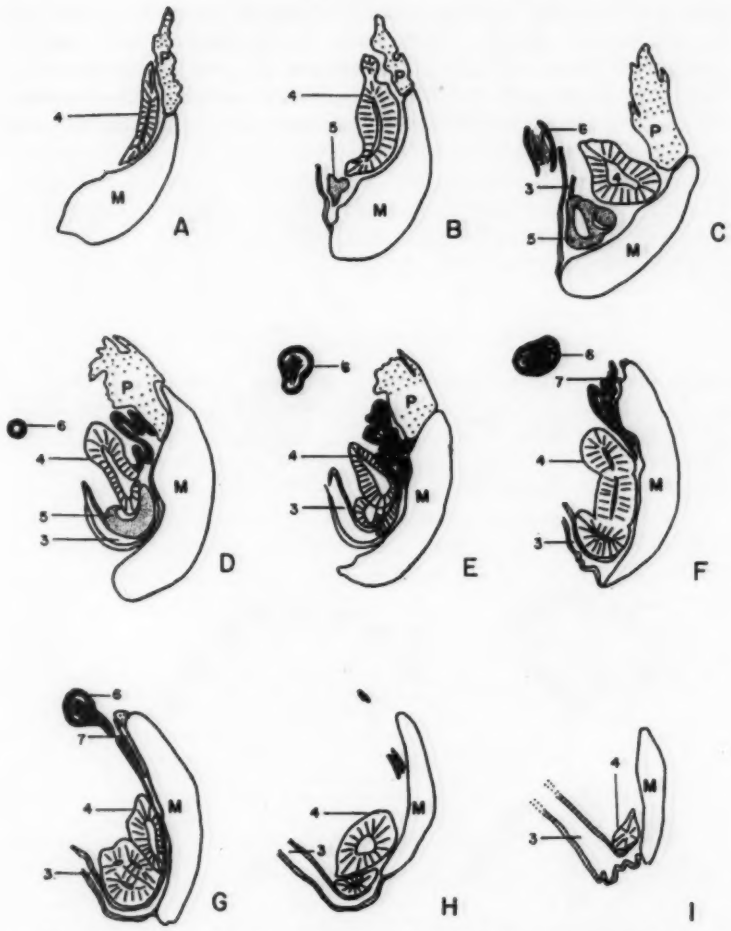

FIG. 5. Diagrams of longitudinal sections of the accessory organs of the reproductive system of *Limacina retroversa* (based upon camera lucida tracings from an animal cut into serial sections which are 5 micra thick).

Figure	*A.*	Section No.	90.	Figure	*F.*	Section No.	155.
	B.	" "	100.		*G.*	" "	165.
	C.	" "	115.		*H.*	" "	175.
	D.	" "	124.		*I.*	" "	195.
	E.	" "	135.				

M. Mantle gland.
P. Penis (in Figs. *A–G*).
3. Receptaculum seminis (Figs. *C–I*).
4. Shell gland (in all the figures).
5. Albumin gland (Figs. *B–D*).
6. Vesicula seminalis (Figs. *C–G*).
7. Out-going duct (Figs. *F* and *G*).

folded, with a thick wall composed of columnar cells, while the second portion is much thinner, being made of cuboidal cells and baggy in appearance (Figs. 4 and 5). Spermatozoa observed in the folded portion stain deeply with iron haematoxylin and are normal in structure, but those within the second portion stain only very lightly with the same

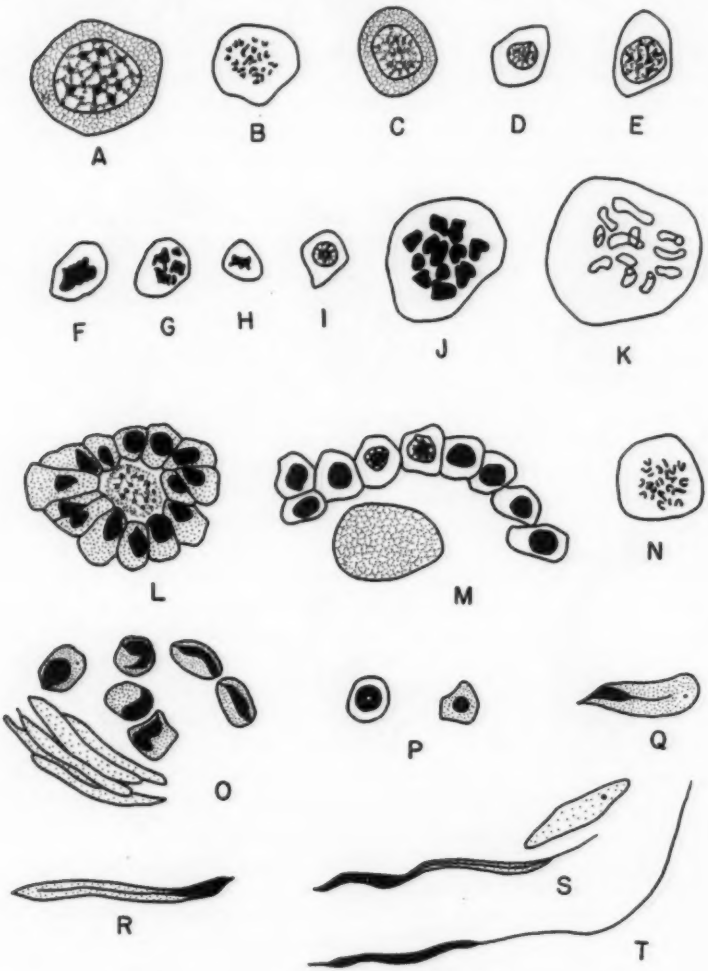

FIG. 6. Spermatogenesis of *Limacina retroversa*.

dye and show signs of degeneration and masses of disintegrating material are often observed.

The penis is practically independent of the rest of the reproductive system both in position and in structure. In well-developed adults it is a very large organ lying dorsad to the rest of the genital structures in the anterior portion of the body near the mantle gland (*P* in Fig. 5). This organ is very much folded on its outer surface when not distended. It has its own secretory glands and retractor muscles. The spermatozoa which come out of the gonoduct run along the ciliated seminal groove towards the penis along the right side of the dorsal region of the "fin" or "flapper."

General Features of Gametogenesis

The development of the male and female reproductive cells has been examined from fixed material cut into serial sections and stained with iron haematoxylin as well as from fresh specimens treated with the carmine smear technique. The steps in spermatogenesis have been ascertained, and it has been found that the maturation of the male germ cells is essentially the same as that of other gastropods.

The pregerminative cells grow and multiply to form the primary spermatogonia (Fig. 6, *A*). The primary spermatogonia (Fig. 6, *B*)

EXPLANATION OF FIGURE 6

A. Primary spermatogonium.
B. Metaphase plate of primary spermatogonium showing the diploid number of 24 chromosomes.
C. Secondary spermatogonium.
D. Primary spermatocyte, beginning of leptotene threads.
E. Primary spermatocyte, showing tetrad condition.
F. Metaphase of first meiotic division, profile.
G. Polar view of anaphase of first meiotic division.
H. Early anaphase of second meiotic division, profile.
I. Young spermatid.
J. Chromosome plate of early anaphase of first meiotic division showing the haploid number of 12 chromosomes.
K. Chromosome plate of early anaphase of second meiotic division, showing 12 chromosomes.
L. A group of spermatids around a central cell.
M. Same as *L*, with spermatids not completely enclosing the central cell.
N. Metaphase plate of a somatic cell, showing the diploid number of 24 chromosomes.
O. Separate spermatids undergoing nuclear elongation, besides them a mass of discarded cytoplasm from other older spermatids.
P. Cross-sections of spermatids showing the central canal.
Q. Elongated spermatid, showing formation of tail filament.
R. Spermatid further elongated than *Q*.
S. Nearly mature spermatozoön with discarded cytoplasm beside it.
T. Mature spermatozoön.

divide by mitosis to give rise to the secondary spermatogonia (Fig. 6, *C*) which are smaller in size and with a higher nucleus-cytoplasm ratio than the primary. Generally the nucleus is placed on one side of the cell. Figure 6, *B* is a metaphase plate of a primary spermatogonium showing 24 chromosomes. The secondary spermatogonia differentiate into primary spermatocytes by mitosis. The primary spermatocytes are identified by the fact that they are nearly always grouped together in the gonad. At the first meiotic division the chromatin of the primary spermatocyte breaks up and forms loose masses and then changes into leptotene threads. These threads, as in some pulmonates, for example, *Succina ovalis, Ostrea lurida,* form such a compact mass that it is difficult to follow the steps in conjugation and tetrad formation (that is, parasynapsis and diakinesis). Figure 6, *D* shows the beginning of the leptotene threads with the nucleolus still visible and Fig. 6, *E* shows a protetrad condition of the chromatin material after parasynapsis. The tetrads of early diakinesis in the form of circles and crosses are arranged on the surface of the nuclear membrane and take a very dark color in staining. A profile of the metaphase of the first meiotic division is shown in Fig. 6, *F,* while Fig. 6, *G* is a polar view of the anaphase of the same division. Figure 6, *J* is a much enlarged polar view of the early anaphase of this division showing the reduced number of 12 chromosomes. During telophase the chromosomes draw together and form a compact mass in the secondary spermatocytes.

From the fact that a resting stage of the secondary spermatocyte is not seen it is inferred that this stage is most probably absent. This situation has been reported by Furrow (1935) for *Valvata tricarinata.* Cells undergoing the second meiotic division are smaller in size than those of the first. Figure 6, *H* is a profile of the early anaphase of the second meiotic division, and Fig. 6, *K* is a much enlarged view from one pole of the chromosome plate at an early anaphase of this division showing the haploid number of twelve chromosomes. During the telophase of this division the chromatin material becomes reticular as the nuclear membrane reappears and later condenses into a very dark staining structure which eventually becomes the dark nucleus of the spermatid. In Fig. 6, *I* a newly-formed nucleus of a younger spermatid is shown. Two of the spermatids of Fig. 6, *M* are also in this condition. These figures show the formation of reticular structures within the nuclear membrane and the condensation of the chromatin material in forming the dense nucleus of the spermatid.

The spermatids are generally arranged around a large degenerating cell which may be homologized with the " nurse cell " reported in most mollusks, but no cytoplasmic connection between it and the spermatids

has been observed (Fig. 6, *L* and *M*). In spermiosis the darkly-staining nucleus elongates first at one point so as to assume an acutely ovate outline (Fig. 6, *L*). A few of the various shapes assumed by the nucleus during its metamorphosis into the head of the mature spermatozoön are shown in Fig. 6, *O*. In association with the change of the nucleus, the cytoplasm elongated and the axial filament of the tail-piece appears (Fig. 6, *Q*). In the cross-section of an old spermatid of this stage the central canal through the nucleus can be seen as a light area in the center (Fig. 6, *P*). The spermatid is transformed into a mature spermatozoön by further elongation of the cytoplasm together with the growth of the axial filament and lengthening of the nucleus followed by a discarding of a greater portion of the cytoplasm (Fig. 6, *Q* to *S*). The head-piece of the spermatozoa is slender and pointed at the anterior end. It is formed by the nucleus. The tail-piece consists of an axial filament and a very slender tail membrane formed by the cytoplasm. The middle piece of the spermatozoön is not clearly seen and its development could not be followed in our material. A mature spermatozoön (Fig. 6, *T*) is about 30 micra long and its head about half as long as its tail-piece. During spermiosis the spermatids move away from a spherical arrangement about a central "nurse cell" and become irregularly placed as seen by a comparison of Fig. 6, *L* and Fig. 6, *O*. When the spermatozoa are mature they are placed closely together side by side with their heads pointing in the same direction, but they do not form "sperm balls" or "sperm morulae." In no case has a "nurse cell" been observed to which the heads of the spermatozoa are attached as has been reported for some pulmonates and prosobranchiates.

In specimens used in this study no atypical spermatogenesis has been observed. The only element in the gonad which might suggest apyrene spermatozoa is that shown in Fig. 6, *O*. It consists of a mass of elongated, enucleated structures which stain very lightly. But it appears more probable that they are discarded portions of the cytoplasm of the transforming spermatids rather than apyrene spermatozoa for no steps comparable to the atypical spermatogenesis leading to the formation of these structures have been observed.

The diploid number of chromosomes is 24. This number was first deduced from observations on the secondary spermatocytes which exhibit the haploid number of 12 chromosomes and was again checked against the chromosome plates of somatic cells. A metaphase chromosome plate of a somatic cell drawn with a camera lucida from a carmine smear is shown in Fig. 6, *N* and it may be compared with the chromosome plate of the mitotic division of the spermatogonium shown in Fig. 6, *B*. The chromosome number was further confirmed by the chromo-

some plates of the second miotic division which also shows (Fig. 6, *K*) the haploid number of 12 chromosomes.

It has been observed in young gonads (Fig. 2, *B*) that there were at first only a few clusters of spermatozoa in the whole organ. As the animal grows the number of clusters of spermatozoa increases and eventually, in a mature *Limacina,* the mass of spermatozoa constitutes nearly all the male tissue in the gonad. It has been reported in other mollusks that each cluster of spermatozoa descends from one primary spermatogonium. If this is also the case in *Limacina,* then it appears from this gradual increase of the number of clusters of spermatozoa with the

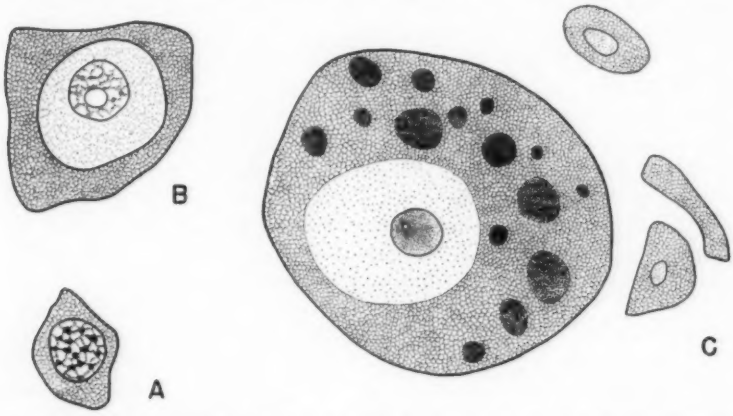

FIG. 7. Drawings of the female reproductive cells of *Limacina retroversa* (Fleming).

A. An oögonium.
B. A growing ovocyte with homogeneous cytoplasm.
C. A nearly mature ovum with a number of darkly-staining granules in the cytoplasm.

growth of the animal that the spermatogonia do not all develop at the same time, but that they follow each other in their maturation.

The female cells as a whole evidently develop slower than the male so that the animal generally assumes a protandric condition. The process of maturation of the female reproductive cells cannot be readily observed in our specimens of *Limacina retroversa.* When the different ovocytes in the ovotestis are compared it is seen that the growth of the cells is associated with the deposition in the cytoplasm of large granules which stain very dark with haematoxylin. The nucleus, on the other hand, takes stain less and less easily and eventually becomes much lighter as the ovocytes grow in size. A mature ovum measures 50 to 70 micra,

which represents an increase of five to seven times in diameter over the first ovocyte. Figure 7 shows an oögonium together with a growing ovocyte and a nearly mature ovum. The change in size is quite noticeable.

As in the case of spermatogenesis, the ova do not all mature simultaneously, but only a few are fully developed at a time. When once started, oögenesis seems to continue throughout the remainder of life of the individual. Female germ cells before and during maturation are located on the peripheral region of the gonad, but ova, when mature, migrate to the central portion of the gonad and thence to the opening of the gonoduct (Figs. 2, *D* and 2, *E*). Fertilization probably takes place during the passage of the ova through the gonoduct.

Female reproductive cells of different ages are present in the gonad, but the maximal number of mature ova observed in a single five micra section of one whorl of the ovotestis is not greater than ten to twenty. The largest number of ova observed in the gonoduct at the time of fixation is about four or five. These observations, together with the fact that *Limacina retroversa* kept in the laboratory lay a few eggs at a time, indicate that this animal does not spawn out after a brief period of intensive reproductive activity, but that spawning is more or less protracted and continuous after it has once began.

Discussion and Summary

From the above description of the maturation of the germ cells of *Limacina retroversa* it will be seen that the process of spermatogenesis in this species agrees with those of other gastropod mollusks. Von Brunn (1884) first observed atypical spermatozoa and considered them to be rudimentary ova. Meves (1903) and Hertwig (1905), however, have described the steps in atypical spermatogenesis and tried to elucidate the morphological and physiological significance of this process and its product. Reinke (1914) in *Strobus bituberculatus* and Kuschakewitsch (1910, 1911) in *Conus* and *Vermetus,* Gould (1917) in *Crepidula plana,* and other authors, made similar studies. Recently Hickmann (1931) described atypical spermatogenesis in *Succina ovalis,* Furrow (1935) in *Valvata tricarinata,* Coe and Turner (1938) in *Mya arenaria.* But as far as it can be ascertained there is no atypical spermatogenesis in *Limacina retroversa.* This species seems to belong to the group of mollusks that do not show atypical spermatogenesis.

Nurse-cells have been reported in many forms of mollusks during their maturation, but in *Limacina retroversa* the arrangement of the spermatids and newly matured spermatozoa about a central cell may

suggest the presence of nurse-cells though no cytological connection has been observed between these and the germ cells.

Mature spermatozoa form balls or "sperm morulae" (according to Orton, 1926) in certain mollusks while in others, though of the same genus, they do not. Thus, *Ostrea lurida* and *Ostrea virginica,* in this country, have clustered and separate mature spermatozoa respectively. *Limacina retroversa* have separate mature spermatozoa.

The accessory organs of the reproductive system of *Limacina retroversa* agree very closely with the description given by Meisenheimer, who did not mention which species were used as representatives of the genus *Limacina* in his comparative studies. However, if Meisenheimer's work on the secondary sexual organs is taken as a typical description of the genus *Limacina,* then *Limacina retroversa* may be considered as closely resembling the typical forms in the structures of the accessory sexual organs.

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THE HISTORY OF A POPULATION OF LIMACINA RETROVERSA DURING ITS DRIFT ACROSS THE GULF OF MAINE

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Geographical distribution in the sea is a hydrodynamic problem, in which the flow of waters determines not only the "climate" of specific regions, but also the supply of organisms to populate these regions. A pelagic population must drift with the waters in which it lives. In contrast to littoral and terrestrial populations, there is no inherent fixity in its occurrence, save that determined by the current systems of the sea. The continuous drift of water through a given region not only raises questions regarding the maintenance of a population there (Damas, 1905; Sømme, 1933, 1934); it renders continuous observation on the natural history of the population singularly difficult, since the individuals observed at any place one day are different from those observed at the same place on another occasion.

Immigrant plankton which do not thrive or reproduce in the Gulf of Maine, but which are carried into its waters from offshore, were found by Bigelow (1926) to occur in a zone extending from the Eastern Channel along the eastern, northern and western sectors of the Gulf as though carried thither by the great cyclonic eddy which Huntsman (1924) and Bigelow (1927) have shown to dominate the circulation of the Gulf. Animals of arctic origin, *Limacina helicina, Mertensia ovum, Oikopleura van hoffeni,* and *Ptychogena lactea* were taken only in the eastern sector of this zone, apparently being unable to survive long enough to be borne further. Other immigrants, both of boreal and tropical origin, survived until carried as far as the offing of Cape Cod, but few were ever taken in the southern sector of the circuit. As his cruises were not taken with sufficient frequency, these observations yield no evidence concerning the velocity of the drift on which these invaders were borne, although its course was clearly enough indicated.

The observations on the pteropod *Limacina retroversa* Fleming [2] de-

[1] Contribution No. 196 from the Woods Hole Oceanographic Institution.

[2] We have employed the name *Limacina retroversa* Fleming because of its current use in oceanography. Dr. W. J. Clench advises me that the generic name

scribed in this paper are of interest because during the greater part of the year it has been possible to follow the history of a rather definite population as it drifted across the Gulf of Maine, noting its rate of spread, its mortality, the growth and reproduction of its individuals, and its replacement by another population of the same species.

Limacina retroversa is a boreal form occurring from latitude 50° to northern Norway off the European coast and from latitude 34° to the southern part of Davis Strait in the western Atlantic. Bigelow considered it one of the most characteristic of the permanent pelagic inhabitants of the Gulf of Maine " where its numbers depend on local reproduction and not on immigration from elsewhere." He also emphasizes the irregularity of its occurrence, an opinion with which I can agree since my observations are at some variance with his. The seasonal variation in the distribution of *Limacina* shown in Fig. 44 of Bigelow's monograph led me to suspect that its occurrence might be closely correlated with the residual drift of the superficial water, since the captures in December and January extend along the northern and western shores of the Gulf, those from February to April occur over the western and southern sectors, while records for May extend from this zone northeasterly toward the Bay of Fundy.

During the years 1933–1934 a systematic survey was made of the Gulf of Maine with the object of obtaining a more precise seasonal picture of its hydrography and biology than was available from the observations of Bigelow. Thirteen cruises were made by the research vessel " Atlantis " in the course of fifteen months, with the result that 684 hydrographic stations in the Gulf of Maine and its adjacent waters were occupied. Whenever weather permitted, a standard vertical haul was made at each station using a Heligoland larva net similar to that described by Künne (1933). The opening of the net was 143 cm. in diameter and it was made of No. 0 silk having 38 meshes to the inch. The net was drawn from a point near the bottom to the surface. The catch of each net haul was preserved and from it the *Limacina* have been separated, counted and measured.

Seasonal Distribution and Abundance

The seasonal distribution and abundance of *Limacina* throughout the year, as shown by the number of specimens caught in each haul, is presented in Figs. 1 and 2. *Limacina* was scarce in the Gulf of Maine

Spiratella has claims of priority arising from Blainville's description (*Dict. Sci. Nat.*, ix, 407, 1817). No attempt has been made to distinguish *L. retroversa* from *L. balea* Möller since the distinctness of these species is in doubt (Bigelow, 1926, p. 116).

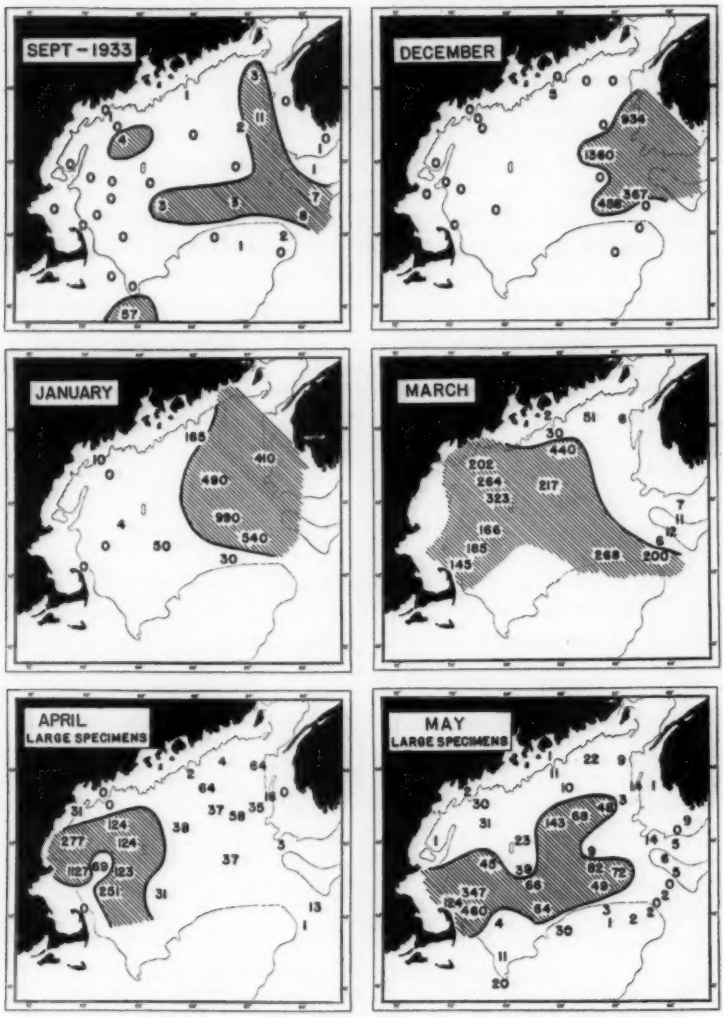

FIG. 1. Charts showing the distribution of *Limacina retroversa* in the Gulf of Maine in September and December, 1933, and in January, March, April and May, 1934. The numbers indicate the positions at which vertical hauls were made and the numbers caught per haul. The shaded area includes all hauls in which the catch was greater than the mean catch for the entire cruise. The numbers entered for April and May include only individuals attributable to population *A* as defined in text.

in the fall of 1933. A dense population of these organisms appeared in the eastern side of the Gulf in December, 1933 and spread westward during the winter. By April these pteropods are concentrated in the western side of the Gulf; the region to the eastward in which they first appeared being occupied by waters scantily populated. As the season advanced the size of the catch at each haul dwindled and the distribution became more general.

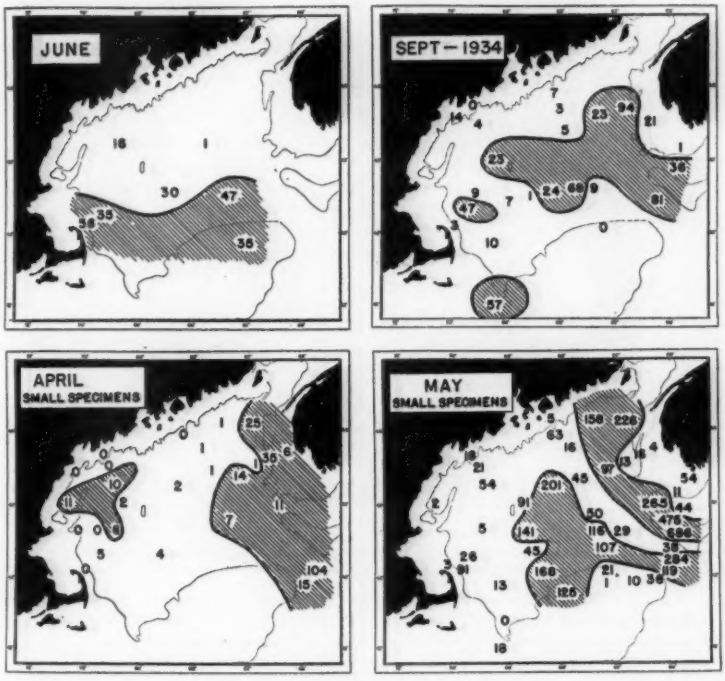

FIG. 2. Charts showing the distribution of *Limacina retroversa* of all sizes in the Gulf of Maine in June and September, 1934, and of small individuals attributable to population *B* in April and May, 1934.

These observations suggest that a huge swarm of *Limacina* drifted into the Gulf from the east in December and were carried westward by the cyclonic drift of water about the Gulf. The acceptance of this interpretation requires evidence on two points: (1) that the population moved not only in the direction but at a rate corresponding to the mass movement of the water, and (2) that a reasonable continuity exists between

the population observed in December in the eastern region and that found in April and May in the western part of the Gulf.

The Circulation of the Gulf

The character of the circulation of the Gulf is adequately described by Bigelow (1927), who with Huntsman showed that the dominant circulation is a great anti-clockwise eddy around the basin of the Gulf,

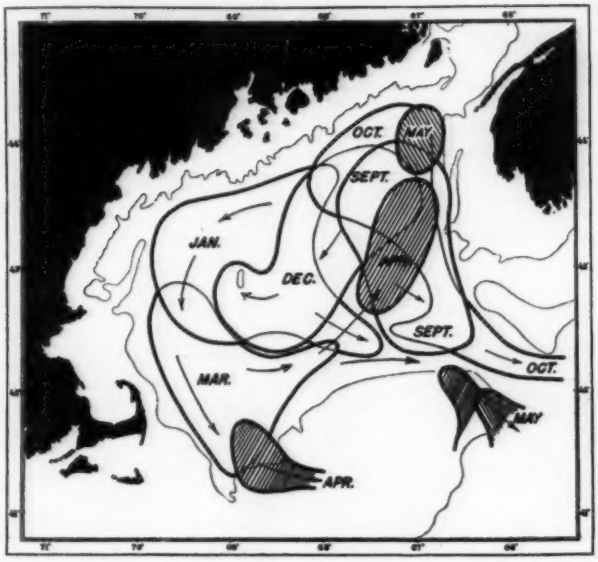

FIG. 3. Positions occupied by an area in which the salinity of the water at 50 meters depth exceeded 33‰ during the period from September, 1933, to May, 1934. Arrows indicate the apparent direction of drift at each period.

varying in velocity and in detail from season to season and complicated by subsidiary eddies. The great eddy receives accessions of water from the east, particularly in winter and early spring; the area of inflow being over the Northern Channel and the adjoining banks. Compensatory losses are occasioned by outflow across the Southern Channel and around the eastern end of Georges Bank. The circulation at the greater depths has a somewhat modified character owing to the confining features of the bottom contour. A report on the dynamics of the circulation of the Gulf, based on observations made during the cruises of 1933–34, is being prepared by Dr. E. E. Watson.

While our vertical hauls have given no indication of the depths at which *Limacina* occurs, it seems probable that it will be carried with the more superficial circulation of the Gulf. Bigelow (1926, p. 121) states that "the most prolific depth zone may be stated as from 20 to 25 meters down to about 80, which corroborates Paulsen's (1910) generaliza-

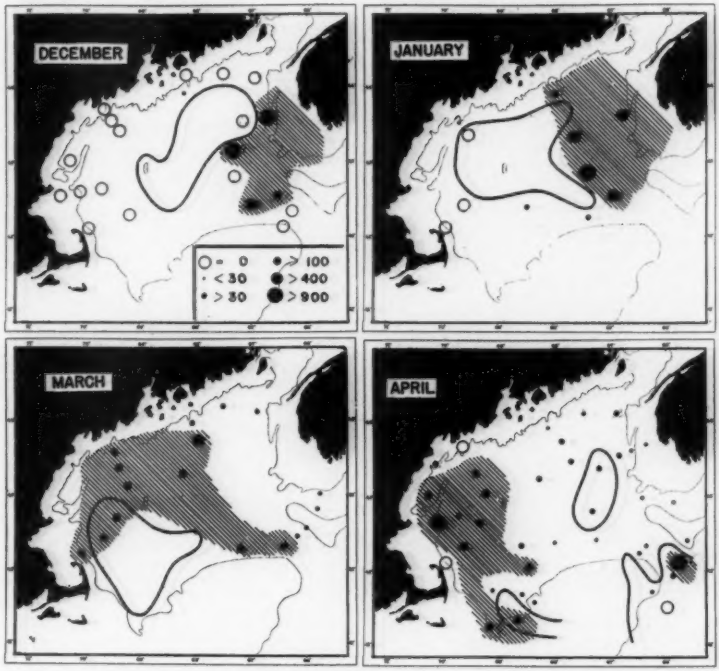

FIG. 4. Charts showing the relative positions of the area of high salinity at 50 meters depth and the distribution of *Limacina* between December, 1933 and April, 1934. The closed contours enclose the area in which $S > 33^0/_{00}$. The hatched areas include catches greater than 100 specimens per haul. The size of each catch is roughly proportional to the area of the black circles. Open circles represent hauls in which no *Limacina* were caught.

tion that *Limacina* lives chiefly shoaler than 50 meters in north European waters, though it has occasionally been taken much deeper." That the sub-surface waters actually drift with velocity as well as direction comparable to that characterizing the *Limacina* population is indicated by a study of the distribution of salinity in the upper water levels throughout the period of the survey. An extensive area characterized

by salinities higher than the surroundings occurred in the eastern part of the Gulf in September,1933. Bigelow (1927, pp. 767, 768) noted a similar occurrence in 1913, 1914 and 1915. The position of this area gradually shifted during the following months, until March when it occurred in the southwestern part of the Gulf. Subsequently it could be traced with less certain continuity as it divided, apparently drifting in part out to sea, and in part in a northeasterly direction. The "saline pool" is most clearly limited in the data for salinities at 50 meters, at which depths it is enclosed by the $33^0/_{00}$ isohaline. The successive

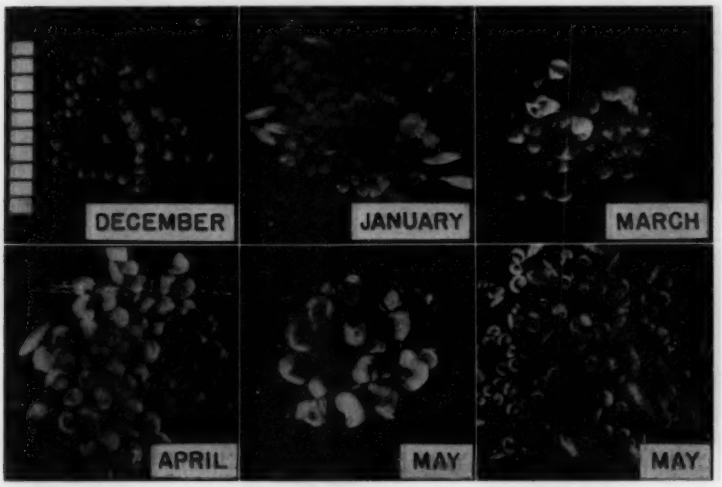

FIG. 5. Photographs of representative catches of *Limacina retroversa* taken between December, 1933 and May, 1934 showing growth. The large specimens taken in May are from the western basin and represent the size attained by population *A*. The small May specimens were taken in the eastern part of the Gulf and are attributed to population *B*. Scale, 1 centimeter divided into tenths.

positions of this isohaline are illustrated in Fig. 3, and correspond closely to the superficial circulation as deduced and illustrated by Bigelow (1927). The velocity of drift of this area, characterized by relatively high salinity, corresponds closely to that of the *Limacina* population which apparently followed close in its rear (Fig. 4). The observations on the distribution of *Limacina* and the occurrence of water of $S > 33^0/_{00}$ at 50 meters depth agree in indicating a drift during the winter period of the sub-surface waters through a distance of 150 miles in four months or at a rate of about 1.25 miles per day.

The Distribution of Size at Different Seasons

The identity of the population sampled at different periods is indicated by an examination of the distribution of size among the specimens taken. These appeared to grow larger as the winter advanced (see Fig. 5). In April and May, however, large numbers of small specimens appeared in certain catches. Since the animals taken at different times and places can only be attributed to the same population if they display a reasonable relation in size, the distribution of size among the individuals of each catch was determined. The largest diameter of the specimens was measured as they lay in random positions in a watchglass. Care was taken to avoid measurements along diameters exaggerated by the spreading of the foot as it protruded from the shell. The measurements were separated into 9 size groups of 0.3 mm. span. The number in each group was expressed as a percentage of the total catch falling within each group.

Figure 6 shows the distribution of size among all the specimens taken during each cruise. In December size distribution is homogeneous; the modal class size is 0.6–0.9 mm. with a uniform distribution of smaller and larger sizes ranging between 0.3 and 1.2 mm. In January the modal class size is the same, but the larger classes have grown at the expense of the smaller. By March a general increase in size is apparent, the modal class being 1.2–1.5 mm. and almost all specimens falling between 0.6 and 1.8 mm. The general shape of the polygon is unchanged as should be the case if the increase in size is due to uniform growth. In April the frequency polygon becomes skewed. Though the modal size is the same as in March, the larger classes have increased in number, relative to the mode, as demanded by growth. The skew is due to the appearance of significant numbers of very small (less than 0.6 mm.) individuals which were not present in the March population. In May these small specimens increase greatly in numbers with the result that the frequency polygon is bimodal. The principal modal size is now 0.3–0.6 mm., being determined by the small individuals. A secondary mode occurs between 1.5 and 1.8 mm., apparently representing the mode of the original population which determined the mode during the previous periods.

The observations made between December and May may be readily interpreted as follows. A homogeneous population of small individuals entered the Gulf from the east in December and grew continuously in size. In April a new population of small individuals appeared and by May these had increased in numbers so as to dominate the size distribution. These may represent a generation of offspring from the original

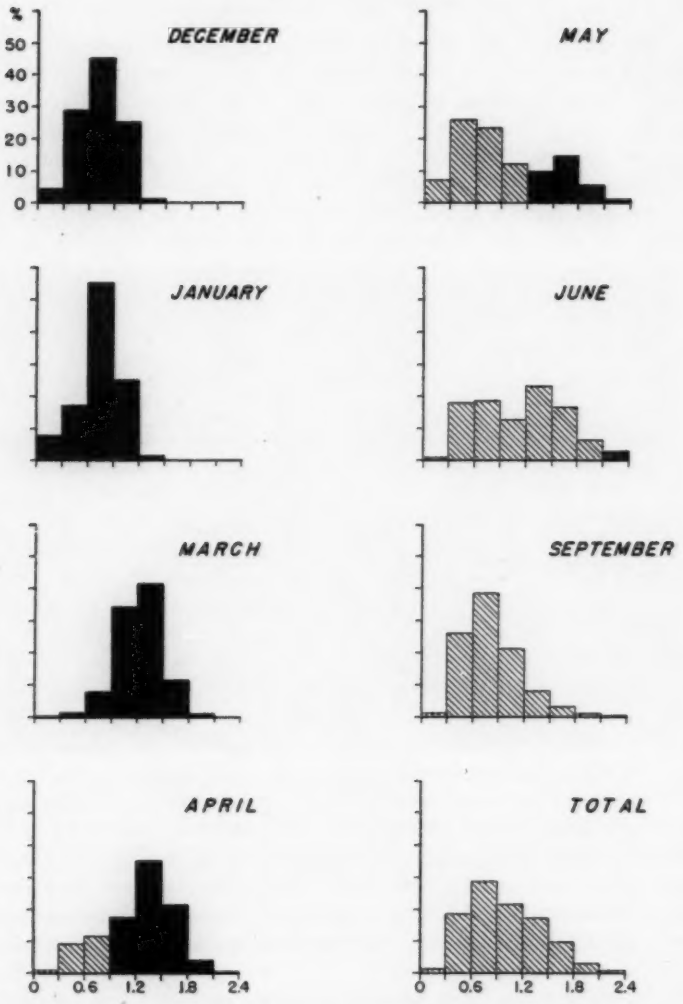

FIG. 6. Frequency polygons showing the distribution of size in the total catch of *Limacina* obtained during each cruise. The lower right-hand polygon represents the combined distribution for the entire series of cruises, corrected for variation in fishing effort. Ordinates, percentage of entire catch falling in each size group. Abscissae, size groups divided in intervals of 0.3 mm. diameter. Black areas are attributed to population *A*.

population, or an invasion of a new group from outside the Gulf, or both. For convenience we will call the original population which appeared in December the *A* population; the new population which appeared in April the *B* population.

The observations made subsequent to May are more difficult to interpret. The frequency polygon for June is again bimodal. The principal size class is now 1.2–1.5 mm. This is smaller than the mode of the *A* population for the preceding month and may be tentatively assigned to the *B* population, which may be expected to grow rapidly with the warming of the water. The largest size class, 2.1–2.4 mm., is relatively more abundant than at any previous time and probably contains the last survivors of the *A* population. A secondary mode occurs between 0.3 and 0.9 mm., which may represent a further production or a new invasion of young individuals, a hypothetical *C* population. The small specimens taken in June were caught a few miles off the coast of Cape Cod. Because of the remoteness of this position from the inflow into the Gulf, they are in all likelihood the offspring of the *A* population, which was richly represented in this region during the preceding cruise, rather than to a new invasion. Only five hauls were made during the June cruise and these all in the southwestern half of the Gulf. The data for this period are not as representative as those obtained at other times.

The scanty and scattered catches made in September, 1934 yield a simple frequency polygon with maximal size class at 0.6–0.9 mm. The distribution of size is generalized and resembles that of the combined measurements of the total collections throughout the year. Apparently during the summer growth is rapid and reproduction and recruitment of the population from without the Gulf is more or less continuous so that separate broods or populations can no longer be distinguished. Dr. Sidney Hsiao has studied the reproductive history of the collections and finds that *Limacina* produce eggs in small numbers continuously after reaching a size of 1.0 mm. It may be inferred that the offspring of one generation will vary greatly in age and after several generations their descendants may be represented by a complete assortment of sizes in which separate broods will be indistinguishable.

The Distribution of Size in the Population of Different Sectors of the Gulf

Let us return to the examination of the history of populations *A* and *B* with a view to determining whether they are actually homogeneous populations and to distinguishing whether population *B* arises through reproduction within the Gulf, or invasion from outside.

The size distribution of representative catches made in different parts of the Gulf at each period has been examined. For this purpose the Gulf has been divided into seven sectors as indicated in Fig. 7. These sectors are arranged in the order in which the non-tidal drift of water may be expected to carry a pelagic population in the circuit of the Gulf. It should be noted, however, that much water will be carried from the

FIG. 7. Chart of Gulf of Maine showing principal place names and the sectors into which the area is divided for analysis of population distribution. Contour encloses depths less than 100 meters.

Yarmouth and Mt. Desert sectors directly to the Georges sector without penetrating the western sectors of the Gulf, as Fig. 3 indicates. Representative catches made at each period in each of these sectors have been selected and their size frequency polygons are given in Fig. 8.

Figure 8 shows that from December through March the size distribution in different parts of the Gulf is homogeneous and that the in-

crease in size noticeable in March is of general occurrence. The extension of the population westward during the winter is apparent. The

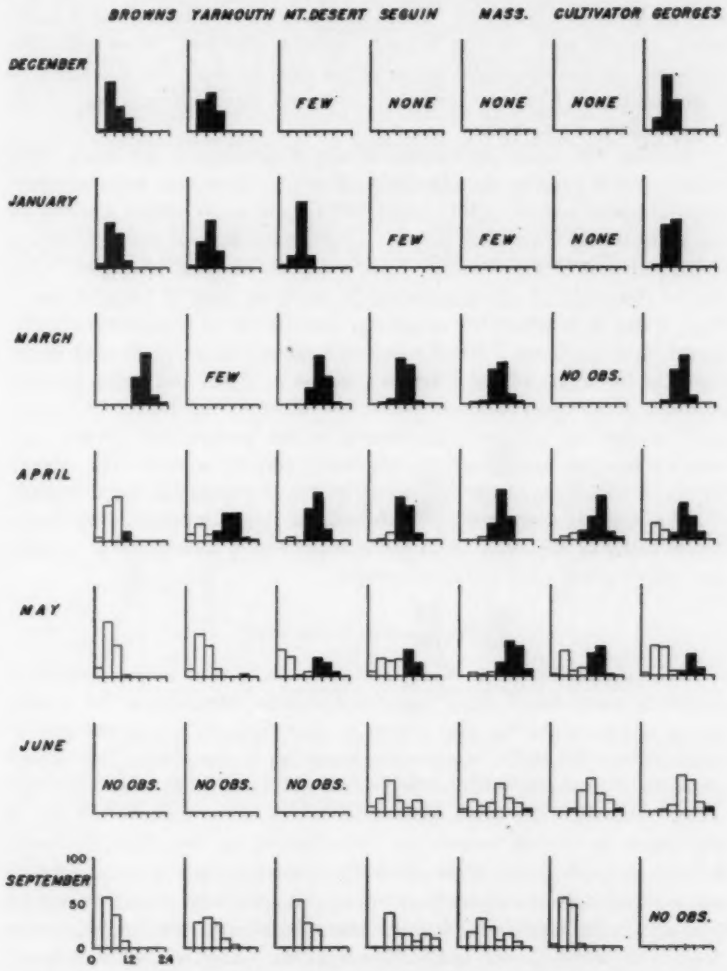

FIG. 8. Frequency polygons showing the distribution of size in representative catches of *Limacina* from each sector of the Gulf of Maine during each cruise. No hauls were made in the sectors marked *No Obs.* Ordinates, percentage of entire catch falling in each size group. Abscissa, size groups divided in intervals of 0.3 mm. diameter. Black areas are attributed to population *A*.

continuous occurrence of *Limacina* in the Georges sector is in accordance with the characteristics of the current system as noted above. In April the homogeneous *A* population appears in all the northern and western sectors of the Gulf. Small individuals representing the *B* population appear now in the eastern sectors, Browns, Yarmouth and Georges. In the outermost sector to the east, Browns, the *B* population is unaccompanied by any specimens of larger size, characteristic of the *A* population at this time.

In May the small individuals of the *B* population are much more widespread, appearing abundantly in all sectors except the most westerly, Massachusetts sector. Only small individuals occur in the stations of the Browns and Yarmouth sector. The populations of the Mt. Desert, Cultivator and Georges sectors have a mixed, bimodal character. The duplex character of the population in April or May is brought out in Figs. 1 and 2, in which the numerical distribution of individuals of different sizes is shown. Small specimens appear in numbers only in the water in the offing of the Eastern Channel in April and occur in small numbers along the course of the inflow through the Eastern Channel. In May they are generally distributed in the eastern part of the Gulf which is most accessible to inflowing water and are scarce in the western region in which the larger specimens of the *A* population are dominant.

These observations justify the belief that the *A* population represents the invasion of the Gulf from the eastward by a homogeneous population which grows as it drifts westward.

Reproduction in the Gulf

The appearance of the *B* population in the eastern sectors, unaccompanied by members of the *A* population and the extension of the smaller group westward during May makes it clear that the new population of small individuals which appears in the spring is due chiefly to a second invasion of *Limacina* into the Gulf from waters offshore and to the eastward. Whether the small individuals which occur mixed with the *A* population in several sectors are the offspring of the *A* population is difficult to decide. Dr. Hsiao finds the *A* population to contain sexually active individuals during the spring, so that offspring from this population are to be expected. On the other hand, the relative scarcity in May of the *B* population in the Massachusetts sector where the *A* population was then most concentrated, argues against the possibility that the *B* population in neighboring sectors is in any important degree the offspring of the *A* population. It is noteworthy that when the *A* population first appeared in December it was dominated by individuals of

small size and no individuals of large size occurred among them. This was also true in many hauls in which the *B* population appeared in April and May. This indicates that the parental generation does not survive after breeding long enough to be caught along with offspring which have grown to diameters of 0.3 mm. or greater. This consideration also supports the view that the hauls in which large and small individuals occur together represent the mingling of populations of different origin rather than the simultaneous presence of a parental and filial generation.

Growth

Since the foregoing analysis of the collections appears to warrant the conclusion that the *A* and *B* populations are homogeneous entities,

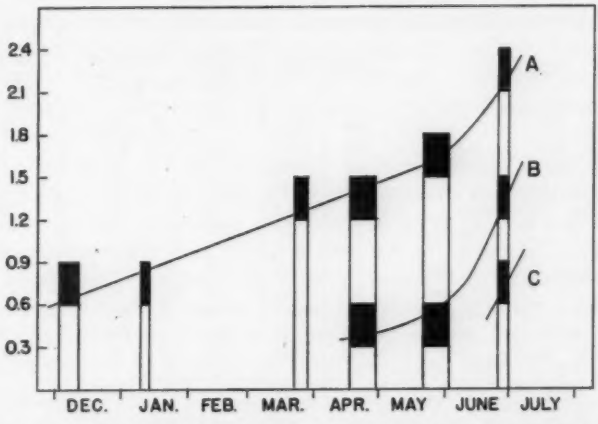

FIG. 9. Diagram indicating the increase in diameter of the modal class of *Limacina* of populations *A*, *B* and *C* during the winter of 1933–34. Ordinates, size groups divided in intervals of 0.3 mm. diameter. Abscissae, time during which collections were made. The black areas represent the modal class size of each population.

we may examine the growth rate of *Limacina* by comparing the size of the modal classes of these populations at different times throughout the year. In Fig. 9 the size of the modal class is plotted against the time the collection was made. Roughly speaking, the diameter of the *A* population doubles in about five months during the winter. At this time the temperature of the water column as a whole is below 7° C. The *B* population observed in May and June grows very much more rapidly as might be expected from the higher temperature of the surface waters at that time.

Numerical Abundance and Mortality

The *A* population is sufficiently established as a distinct and homogeneous entity and was under observation for a sufficiently long period to warrant some statistical examination of its numerical abundance. It must be realized that the number of hauls made in each cruise is insufficient and their distribution too irregular to allow great confidence in the outcome of the analysis of a matter of such complexity, but data of this sort are so rare and so difficult to obtain that one is warranted in the attempt to get the utmost out of it.

TABLE I

Statistics of the size of catches of Limacina retroversa made during the cruises of 1933–1934.

Cruises	No. of Hauls	Total Catch	Mean Catch per Haul	Per Cent of Hauls with Limacina Present	Per Cent of Hauls with Catch Greater than Mean
Sept. 2–14, '33	34	104	3	44	21
Dec. 2–11, '33	22	3120	142	23	18
Jan. 8–13, '34	12	2685	224	66	33
Mar. 21, 29, '34	18	2535	141	100	55
April 17–23, '34*—Small	24	141	6	71	33
Large	24	2575	107	84	24
Total	24	2716	113	100	23
May 21–June 3, '34—Small	44	4068	92	98	25
Large	44	1849	42	91	25
Total	44	5917	134	100	23
June 25–July 1, '34	7	220	31	100	57
Sept. 17–27, '34	26	507	20	92	20

* Two hauls made in the Eastern Channel on May 6, 1934 are included in the data for the April cruise.

We may consider that all the individuals taken in December, January and March belong to the *A* population. In order to eliminate as far as possible the *B* population individuals smaller than 0.9 mm. are eliminated from the data for April and smaller than 1.2 mm. from that for May. The *A* population cannot be identified with assurance in the June collections.

Increase in numbers can be due only to the invasion of the Gulf by new immigrants from offshore since the elimination of the *B* population precludes reproduction as a means of increase. Decreases in numbers may be due to water movements, carrying a part of the population out of the area under observation, or to mortality. Since the population first appears densely distributed at one side of the Gulf, some redistribu-

tion of density between different points of observation is to be expected as the result of lateral mixing of the water. This may be expected to result in an increasing uniformity of distribution.

Table I contains some statistics concerning the size of the catches made during the several cruises. An indication of the numerical abundance of *Limacina* in the Gulf as a whole at each period is given by the numbers in column 4 showing the average number taken per haul. Between December and January the numbers increase, after January there is a progressive decline in abundance. An examination of the distribution of *Limacina* from month to month (Fig. 1) warrants the conclusion that the invasion of the *A* population into the Gulf continues through December and January since large catches are made at these times in the region of inflow. The percentage of stations at which *Limacina* was taken (column 5) increases three-fold during this interval. The invasion appears to terminate in January, since following that period no considerable numbers of individuals referable to the *A* population were taken in the region of inflow into the Gulf. Invasion may consequently be excluded as a significant factor affecting the numbers of the population after January.

The numbers of the population decline steadily from January through May, the mean catch of large individuals referable to population *A* decreasing to less than 20 per cent of the original in four months. Significant losses are attributable to the drift of water out of the Gulf. The continuous occurrence of catches containing *Limacina* in the Georges sector, which lies in the path of outflow through the Eastern Channel, and the concentration of the *A* population in the western and southern quarters of the Gulf in April and May, where it is subject to drift outward by way of the South Channel, favor such losses.

It is probable that large losses in numbers also occur as the result of mortality. Measurements of the total volume of zoöplankton caught throughout the Gulf indicate a general mortality of about 60 per cent during the winter period.

The data in Table I indicate that the percentage of stations at which *Limacina* occurred is not less in April and May than in January. If the circulation into and out of the Gulf were rapid enough to account for the greater part of the loss in numbers, a greater decrease in the area of distribution would be expected. It may be argued that since the area of distribution does not decrease while the numbers caught per station decline five-fold, the decline is due to the death of a large part of the population. This argument, however, must be accepted with great reserve, since the lateral mixing of water may cause small numbers of

animals to be carried into regions in which they did not previously occur, and will tend to enlarge the area of occurrence.

Another approach to the question of mortality may be made through an examination of the distribution of size in the catches as a whole throughout the year. In Fig. 6 the size distribution of the *total* catch is represented. This frequency polygon was prepared by averaging the percentages of the population represented by each size group during each period of collection, a procedure which corrects for the variation in "fishing effort" during different cruises, but does not correct for the irregularity of spacing of the cruises in time. The figure shows that the size most frequently caught is 0.6–0.9 mm. Smaller sizes are less frequent in part because both the *A* and *B* populations are carried into the Gulf half-grown, in part because they are more readily overlooked in sorting the catch and in the case of the smallest class, because many may pass through the mesh of the net. The larger size classes diminish progressively in their relative abundance, as would be expected if the population is subject to continuous mortality. The mode of the *A* population is 0.6–0.9 mm. in January, 1.5–1.8 mm. in May. Specimens of the latter size occur about one-third as abundantly as the former in the catches as a whole. This suggests a mortality of about 66 per cent in the time taken to grow from the former to the latter size, during four winter months. Such statistics, however, are not very convincing since the smaller classes are recruited by invasion and the chance of loss by drifting out of the Gulf increases with time, as does the size of the individuals of the population.

Lateral Mixing

The horizontal mixing of water masses, which has recently attracted the attention of hydrographers, may be expected to have consequences of importance in determining the distribution of organisms. If attention is limited to the consequences of steady residual currents, one can only conclude that the pelagic inhabitants of a region such as the Gulf of Maine which is the seat of a cyclonic eddy must be constantly washed away by the flow of water through it. The rise and decline of the *Limacina* population which we have observed supports this conclusion. The existence of lateral mixing, if of considerable extent, should tend to diminish the tendency of residual currents to transport all of the pelagic population out of such a region. By the transport of a part of the population backstream, or around and across the vortex of an eddy, an opportunity would be afforded for the endemic occurrence of the population in spite of the continuous drainage occasioned by the residual currents.

The effect of lateral mixing must tend continuously to make the distribution of the population more homogeneous. After the introduction of a limited water mass containing a rich population into a large unpopulated region, if lateral mixing alone were in operation one would expect the limits of the populated waters to become less distinct and the distribution to become more general. If samples were taken at random at any time during the mixing process, at first a limited number of samples would contain numbers far in excess of the mean value for the collections as a whole, and the remainder would contain small numbers or none at all. As mixing proceeds, the poorly populated waters would become enriched at the expense of the densely populated regions. In both regions the density of population would change in the direction of the mean population for the entire region and eventually the entire distribution would assume a random character in which the modal population density would correspond with the mean. At this point half the samples would contain more than the mean number, and an equal number less than this value. Subsequent mixing would tend only to diminish the variation of the numbers secured in the different samples.

The catches of *Limacina* have been examined for such evidences of lateral mixing. Examination of the distribution of the population shown in Fig. 1 indicates that the populated region was sharply delimited in December—no *Limacina* occurring in regions immediately in advance of the populated waters. In January moderate catches were made in regions well in advance of the main population, suggesting a gradual disintegration of the boundary zone by lateral mixing. In March the area of intense population covers more than half the Gulf and at the same time the numbers present at each station have diminished and become more uniform as required if lateral mixing is in effect. The same tendencies continue in April and May and are particularly evident when the distribution of small specimens (population *B*) is compared. While previous to March less than half the hauls have contained more than the mean catch for the Gulf as a whole, by March at least half the hauls are of this size—as required by random distribution. One feature of the distribution of large specimens in April and May is of particular interest. In the east central part of the Gulf, on the line between Cashes Ledge and Yarmouth, Nova Scotia, the catches of large specimens are on the whole greater in May than in April in spite of the fact that some mortality is doubtless occurring during this period and the movement is contrary to the expected drift of the water.

As a further test to see if the distribution of population is becoming more homogeneous, the catches have been grouped into classes according

to the number taken per haul and the number of catches in each class has been expressed as a percentage of the total number of catches made during the cruise in question. The result is presented in Fig. 10. The left-hand diagram shows the result when the size of the catch is noted

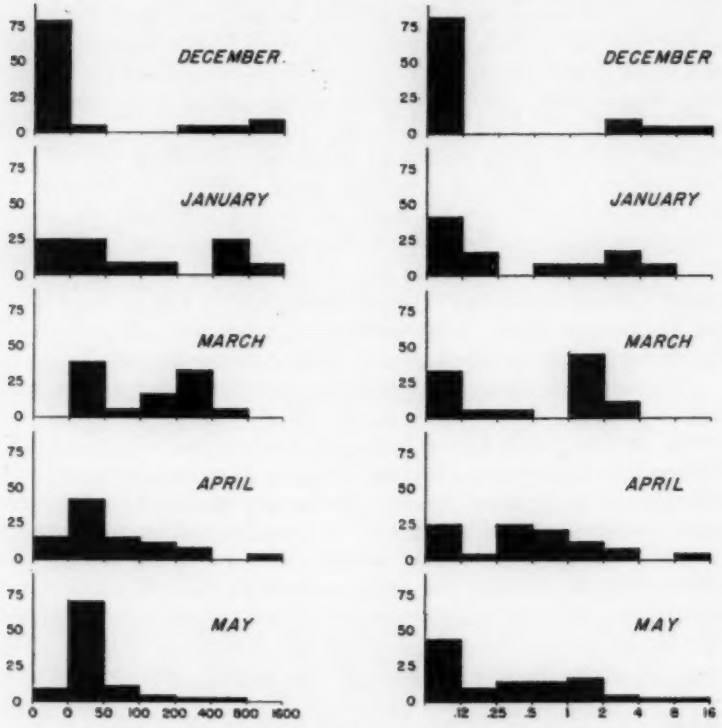

FIG. 10. Frequency polygons showing the distribution of catches of different numerical abundance among the total number of hauls made during each cruise. Ordinates, percentage of entire number of hauls yielding catches of each size. Abscissae, left-hand diagrams, size groups divided in intervals according to number caught in each haul; right-hand diagram, size groups expressed as multiples or fractions of the mean catch per haul during each period.

numerically (and assigned to classes which for convenience increase in geometrical progression). The right-hand diagram represents the same data arranged in size classes which increase as multiples, or decrease as fractions, of the mean population of the region as a whole at the period of collection. The latter procedure eliminates the effect of losses

through mortality. Both diagrams indicate the complete heterogeneity of the samples in December and January and the progressive approach of both the smaller and larger catches toward the mean value which reaches its culmination in April and May.

Actually the situation observed is complicated by the drift of the population across the region and by the invasion of the eastern part of the Gulf by water poor in *Limacina* of population *A* in March and April. This inflow tends to decrease the homogenity of distribution and accounts for the increase in the proportion of catches in April and May which contained few or no *Limacina*. It also explains why the percentage of hauls in which the catch was greater than the mean diminished in the months subsequent to March. These tendencies during April and May at least make it clear that lateral mixing is not sufficiently great to obliterate the effect of the residual current in depleting the *Limacina* population of the eastern portion of the Gulf.

Because of the impossibility of separating the effects of mortality and of losses and gains through the residual movement of the water into and out of the Gulf from the apparent effects of lateral mixing, any attempt to evaluate the extent of the latter has been abandoned. Because of the great influence which such mixing would have in enabling a population to maintain itself in a region from which the residual current would constantly tend to remove it, it seems desirable, however, to emphasize the possibility of such mixing and to indicate the way in which it appears to manifest itself.

Discussion

The evidence seems definite that the population of *Limacina* which occurred in the Gulf of Maine in 1933–34 was not endemic, as Bigelow thought, but owed its origin to two separate invasions of young individuals from offshore. Unlike the less successful immigrants of arctic or tropical origin, *Limacina retroversa* thrives well enough to grow to sexual maturity and perhaps to reproduce within the Gulf and to be carried in considerable numbers to all its parts. For this reason Bigelow obtained evidence which led him to conclude the species was endemic. By following the history of these populations closely, however, it is shown that neither invasion was able to maintain its numbers in the Gulf—either because reproduction was not successful or was inadequate to balance the depletion due to mortality and the drift out of the Gulf.

The two invasions consisted of dense swarms of small individuals of very uniform size, sharply separated by a period when practically no *Limacina* occurred in the region of inflow into the Gulf. The discon-

tinuous character of these invasions no doubt accounts for the irregularity in occurrence of *Limacina* in the Gulf noted by Bigelow. Why the invasion occurs in such definite periodic swarms can be answered only by studies carried out in the waters to the eastward from which these invasions undoubtedly come.

It is an interesting question why the invading swarms are composed of individuals of such uniform size. In describing the population found in the Gulf in September, 1934, it was pointed out that animals of all sizes were mixed together and that successive broods can no longer be distinguished. Dr. Hsiao's studies indicate that the mature *Limacina* continue to produce eggs during a considerable period of growth. Yet we found evidence to suggest that the parental generation does not survive after breeding long enough to be caught along with its offspring at the time when these enter the Gulf. Dr. Hsiao has measured numerous catches of *Limacina* taken over the continental shelf between Cape Cod and Cape Hatteras. Usually these catches are composed of individuals of uniform size, just as were those which invaded the Gulf. This riddle also can be answered only by closely timed observations in the offshore areas of production.

The most conspicuous result of this study is the demonstration of the degree to which the occurrence of *Limacina* in the Gulf of Maine depends upon the circulation of its waters. Damas in 1905 raised the question: How does the plankton of a given region maintain its character in the face of the continual circulation of the currents and how does a given species persist so as to possess a special geographic distribution? He concluded that there must exist a special zone or center of production in which adults abound and reproduce successfully and that to this region circulatory currents serve to bring back periodically a proportion of the individuals which become entrained and dispersed by the continual movements of the water. The circulatory system of the Norwegian Sea subsequently described by Helland-Hansen and Nansen (1909) appeared to supply just such a mechanism as was required to account for Damas' observation of the production centers of copepods in that region. Sømme (1933, 1934) has shown how such centers of production are established for *Calanus finmarchicus* and *hyperboreus* in the deep waters of the Norwegian fjords in winter, and has elucidated the mechanism which results in the release of the organisms from these regions at the time of reproduction. The observations on *Limacina* have not revealed the presence of a center of production in the Gulf of Maine. They point to the existence of such regions offshore to the eastward and are of interest rather in telling something of the fate of these animals, entrained in the movement of water, which are carried away

never to return, yet for a while to occupy an important rôle in the ecology of other regions. Behind the geographical distribution of each species of plankton there must be a complex balance of biological and physical factors. Of the latter, flow of water appears to be paramount; its consequences too frequently neglected.

Summary

A population of small specimens of the pteropod, *Limacina retroversa,* appeared in the eastern part of the Gulf of Maine in December, 1933. From collections made during the following 9 months information was obtained showing that the population was a homogeneous one, that its members grew to maximum size in 5 months, declining in numbers as they did so.

A second population of small individuals appeared in the Gulf in late spring, originating chiefly from offshore, but possibly in part being offspring of the original population. These were unsuccessful in maintaining their numbers throughout the summer.

In addition to the information on the life history of *Limacina,* the data indicate the rate of drift of the water in its circuit of the Gulf. It supplies also suggestive information on the dispersal of organisms through the lateral mixing of water. It emphasizes the dependence of pelagic organisms upon the current systems of the ocean and the difficulty involved in maintaining a permanent population in any one locality.

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RESPONSES OF THE SWIMBLADDER OF THE GUPPY, LEBISTES RETICULATUS, TO SUDDEN PRESSURE DECREASES

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Most fishes maintain a density equal to that of the surrounding water. This is done through a very accurate control of the volume of their swimbladders by appropriate gaseous exchange between the swimbladder and the blood. As the fish passes into deeper water the increased hydrostatic pressure compresses the bladder gases to the point that the fishes are no longer buoyed up in the water. Consequently, in order for the fishes to adapt themselves to the new pressure, they must put more gases into the bladder. Conversely, as the fishes rise in water the decreasing hydrostatic pressure renders the gases in the bladder too buoyant and provision is made for the release of the proper amount. Many experiments have been performed in an attempt to determine the mechanism of control of the swimbladder. The great majority of these experiments have involved analysis of gases found in the swimbladder in different states of adaptation and under controlled experimental conditions. Although much valuable information has been obtained, our picture of the mechanism is by no means complete.

Von Ledebur (1937) summarizes our present knowledge in this field and reviews briefly all the current theories to explain the secretion of gases into the swimbladder against the diffusion gradient and their removal. The variety of explanations indicates the need of more research before a definitive theory can be devised.

The experiments to be described in this report were carried out upon the guppy, *Lebistes reticulatus,* a physoclistous fish. They demonstrate the responses of the swimbladder to decreased hydrostatic pressure and to situations in which the normal diffusion gradient, favoring passage of gases out of the bladder, is experimentally reversed and varied in steepness. Some interesting information has been obtained upon the mechanism of gaseous exchange between the environment and swimbladder.

The apparatus consisted of a two and a half gallon carboy. Through the stopper was projected a small bore glass tube which passed into a flask serving as an air cushion. To this flask was attached a mercury manometer and tubes which led to a vacuum pump on the one hand and

a compressor on the other. Both of the latter tubes were equipped with stopcocks. The whole system was arranged so that the pressure within the system could be rapidly changed but always kept under complete control. The experimental animal was placed in the carboy completely filled with water which rose three or four inches into the small bore glass tube of the stopper. In a few experiments where the gases present in the water were to be equilibrated to each new pressure by shaking, a liter bottle half full of water was substituted for the carboy.

With this equipment it was possible either to alter the pressure upon the fish without appreciably altering the gaseous concentration of the medium,[1] or to subject the animal to atmospheric pressure in water which had been equilibrated with air under heightened or lowered pressure.

With this same apparatus it was a simple matter to measure any changes in the total amount of gases in the bladder by the application of Boyle's law. The method was merely a refinement of the technique used by Evans and Damant (1928). From time to time the exact pressure to which the guppy was adapted was measured. As the amount of gases in the bladder increased, proportionately more pressure had to be applied in order to return the fish to the same density as the water, and conversely, as the fish permitted gases to escape from the bladder, proportionate decrease in pressure was necessary to adjust artificially the fish density so that the fish would tend neither to rise nor sink. The validity of this method depends upon the assumption that the pressure of the bladder gases results solely from the external environment about the fish. Evans and Damant found this to be the case with the physoclistous fish they examined, the bladder gas not being held under pressure by any structure of the fish body.

Some initial experiments were performed with completely normal guppies, but it was found difficult to make accurate readings of the exact pressure to which the fish was adapted at any given moment. This difficulty was a result of the fish's being able to maintain itself quite stationary in the water through an appreciable range of pressures by very slight activity of the pectoral fins. Consequently, all experiments reported here were conducted with fishes whose pectoral fins were amputated. This last procedure permitted equilibrium readings of considerable accuracy in a few seconds of time. There has been no reason to suspect that this fin removal affects the swimbladder response in any manner.

Such a method as the one used here may be applied to any physoclistous fishes since in these fishes the change in gas quantity takes place

[1] When not otherwise specified in the following experiments, the gaseous content of the water was that resulting from equilibrium with air at room temperature and the atmospheric pressure (about 750 mm. Hg).

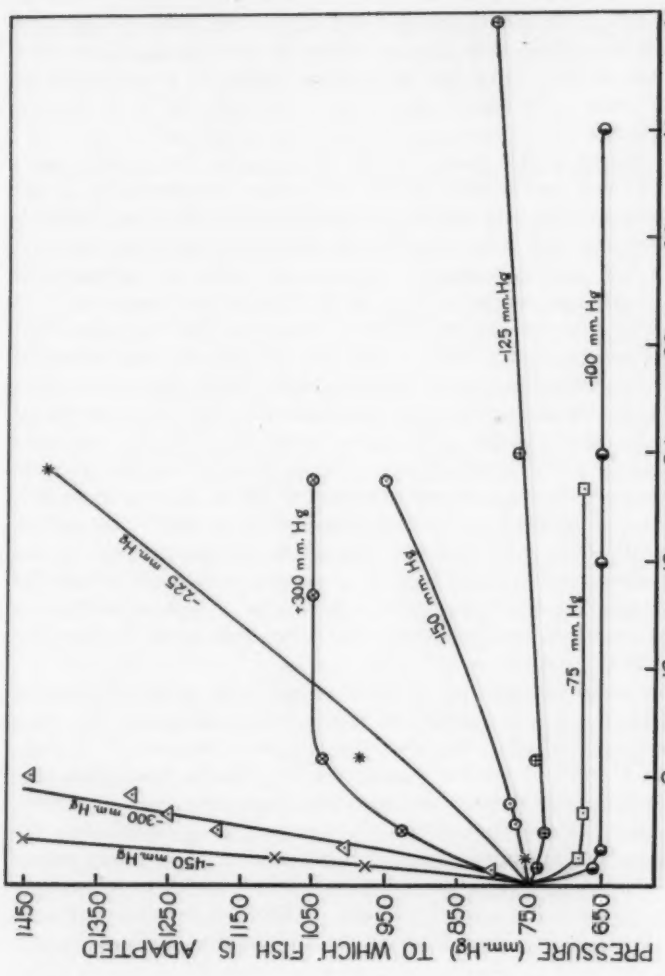

FIG. 1. Diagram showing the responses of the swimbladder to decreases in pressure of 75, 100, 125, 150, 225, 300, and 450 mm. Hg, and an increase of 300 mm. Hg.

only very slowly by secretion or diffusion of gases and hence the few seconds of sudden change of pressure for the purpose of determining the direction and extent of gas change cannot result in an abrupt change in gas content. Physostomous fishes such as the goldfish, on the other hand, almost instantly discharge gas bubbles by way of the mouth when the pressure is suddenly decreased.

Experimental

The first experimental series was designed to test the effect of changes in pressure upon the fish swimbladder response. Water was equilibrated with air at room temperature and at atmospheric pressure and placed in the carboy. A female guppy was permitted to adjust its density to that of the water in the new situation: a pressure of one atmosphere plus eighteen inches of water. This situation served as a starting point for each experiment. In as many separate experiments the pressure on the fishes was decreased by 75, 100, 125, 150, 225, 300, and 450 mm. Hg and increased by 300 mm. Hg. The responses of the fish to these changes are indicated in Fig. 1.

The fishes responded to increased pressure by decreasing their density gradually over the course of six or eight hours to the point of equalling that of the water. This density was maintained as long as the pressure was held constant. When the pressure was decreased by 75 or 100 mm. Hg, the fish increased its density to a value equal to the surrounding water.

However, as the pressure was decreased more, by 125, 150, 225, 300, and 450 mm. Hg, the fish no longer adaptively adjusted its density. Instead, its density decreased at a rate that was obviously a function of the amount of decrease in pressure. These results were confirmed by a second experiment in which only male guppies were used.

With pressure decreases of more than 100 mm. Hg the density of the fish continued to decrease for several days at a nearly constant rate, or until the swimbladder became so large that the wall of the visceral cavity was distended in a balloon-like fashion. When the pressure was decreased by 300 or more mm. Hg such a state was reached in less than twenty-four hours.

There thus appeared to be some level of pressure below which the fish was unable to adapt itself, and on the contrary, became more and more maladjusted. This pressure level appeared to be quite constant whether it was reached in one sudden pressure change, in two equal changes over several hours, or in three changes over a period of four days. Figures *2A* and *2B* illustrate results of this nature that were

obtained. Furthermore, the rate of decrease in fish density in response to a decreased pressure appeared to be independent of the rate at which the value was approached.

Interpretation of the Experimental Results

The cause of the abrupt reversal of fish response from density increase to decrease as the pressure was decreased by values greater than

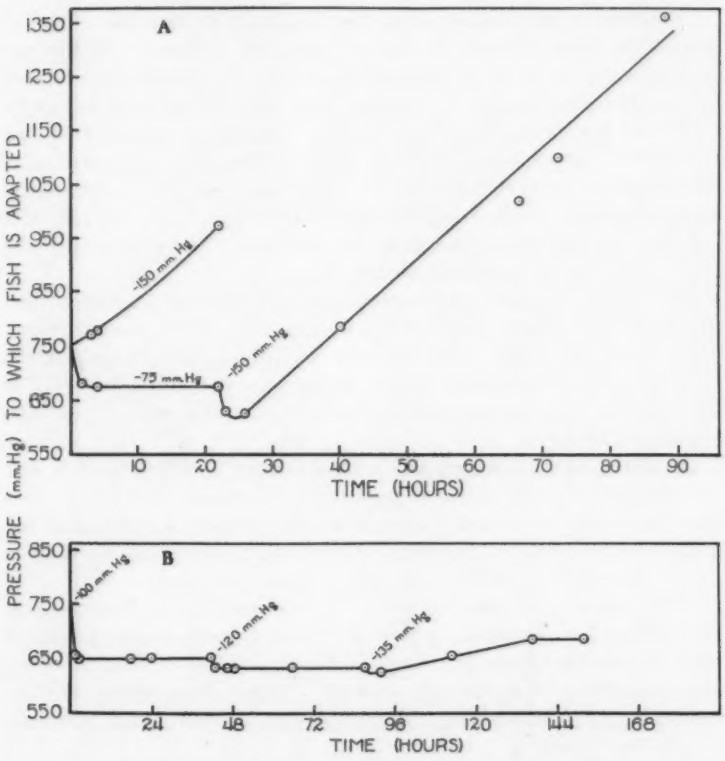

Fig. 2. (*A*) Graph showing the swimbladder responses to reduction in pressure when the reduction is carried out in a single step, in two steps over a period of about 20 hours, and (*B*) three steps over a period of about four days.

about 120 mm. Hg was the first question to be answered. A guppy adapted to a pressure increase of 300 mm. Hg, when suddenly returned to the original pressure, responded by increasing its density to a value

again equal to that of water. On the contrary, a guppy directly subjected to a similar percentage decrease in pressure responded in an opposite fashion by decreasing its density. Therefore, a physically induced size increase of the swimbladder and any activity or response of the fish directly correlated with such increase would not totally explain the situation. This eliminated any reflex associated with the eyes, fins, etc., as was indicated by Meesters and Nagel (1934) to be true over the normal pressure range.

A brief additional experiment was performed which immediately eliminated any induced change in amount of gases dissolved in the water as stimulating the bloating phenomenon. A guppy in an unstoppered flask of water was placed in a vacuum desiccator. The pressure on the fish was then decreased by 300 mm. Hg. The fish commenced to bloat. The water now had a dissolved air content found in conjunction with a decreased pressure which stimulated the bloating of the fish. The flask was quickly removed from the desiccator and paraffin oil poured upon the water surface to prevent any re-diffusion of gases into the water at the restored pressure. If decreased concentration of any gases in the water was responsible for the bloating phenomenon, such bloating would be expected to continue. The fish in this situation increased its density to a value equal again to that of water.

It was highly improbable that the fish was responding to pressure through the activity of any sense organ. The evidence indicated that if a postulated hydrostatic pressure sense organ was actually operating it displayed no signs of adaptation or fatigue (see especially Fig. 2*B*). A physical explanation thus appeared more plausible.

Two further and decisive experiments were performed which demonstrated the character of this pressure effect. A guppy was placed in a bottle only partially filled with water. The water was thoroughly shaken with air at each reduced pressure. The fish adjusted its density to agree with that of the water for all the pressure decreases used. In one experiment the pressure was decreased by 300 mm. Hg. Again, when water was thoroughly shaken with air under a pressure increase of 500 mm. Hg, the guppy bloated when placed in this after the pressure had been restored to the original value. The rate of bloating was of the same order of magnitude as seen in response to a pressure decrease of 300 mm. Hg.

A partial interpretation of the experimental results thus seems evident. Except for the pressure exerted by the short column of water over the fish in the experiments, the gas in the bladder is of the same pressure as the atmospheric air at the water surface. Under reduced pressure, tension of gases in the water favors diffusion of gases into the

bladder and atmosphere alike. The greater the decrease in pressure, the more rapid the passage of gases into the bladder.

Not up to this point explained, however, is the initial lower rate of bloating, sometimes even temporary gas output, which occurred when guppies were subjected to decreases in pressure sufficiently large to produce the bloating phenomenon. The temporary output is seen in Fig. 1 (reduction in pressure by 125 mm. Hg) and in Figs. 2*A* and 2*B* at decreases in pressure by the amount 150 (when this was carried out in two steps) and 135 mm. Hg. This temporary initial activity, an apparent attempt of the fish at adaptation, commences strongly and then gradually over the course of two to five hours becomes weaker until it is completely masked by the inward passage of gases. Either an outwardly secreting mechanism which could secrete quite efficiently up to a point of fatigue, or simple diffusion alone, might account for the response. The former explanation can neglect the partial pressures of the various gases within the swimbladder. The latter explanation is based upon the assumption that at the beginning of the experiment the bladder possesses a higher percentage of CO_2 than does air.

Recent work of Meesters and Nagel (1934) has shown that a fish would, under the appropriate stimulation, decrease the amount of bladder gas most rapidly when the initial percentage of CO_2 in the bladder was high. Thus, fish were apparently able to eliminate this gas much more rapidly than either O_2 or N_2. The work of Jacobs (1932), confirmed by Meesters and Nagel (1934), has demonstrated that a physo-

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FIG. 3. Responses of the swimbladder to sudden decreases in pressure following immediately upon different experimental conditions of secretion and diffusion of gases to and from the bladder.

A. Two experiments in which guppies are subjected to a pressure decrease of 300 mm. Hg in water equilibrated with air at the reduced pressure, then returned to atmospheric pressure in water equilibrated with the restored pressure. At the moment of adaptation to the restored pressure the fishes are subjected to a sudden pressure decrease of 300 mm. Hg.

B. A single experiment in which a guppy was subjected to pressure increase of 260 mm. Hg and at the moment of adaptation subjected to a decrease to a point 300 mm. Hg below atmospheric pressure.

C. Three experiments in which guppies long (20 hours to several days) adapted to a given pressure are subjected to a sudden pressure decrease to a point 300 mm. Hg below atmospheric pressure.

D. Two experiments in which guppies are stimulated to decrease the gas content of their bladders and then suddenly subjected to a pressure decrease to a point 300 mm. below atmospheric pressure.

E. The two upper graphs indicate the response of guppies, bloating as a result of a 300 mm. Hg decrease in pressure, when they are suddenly returned to atmospheric pressure. The lower graph shows the normal response of the fish upon increase in pressure of 300 mm. Hg and then, after some hours, return to atmospheric pressure.

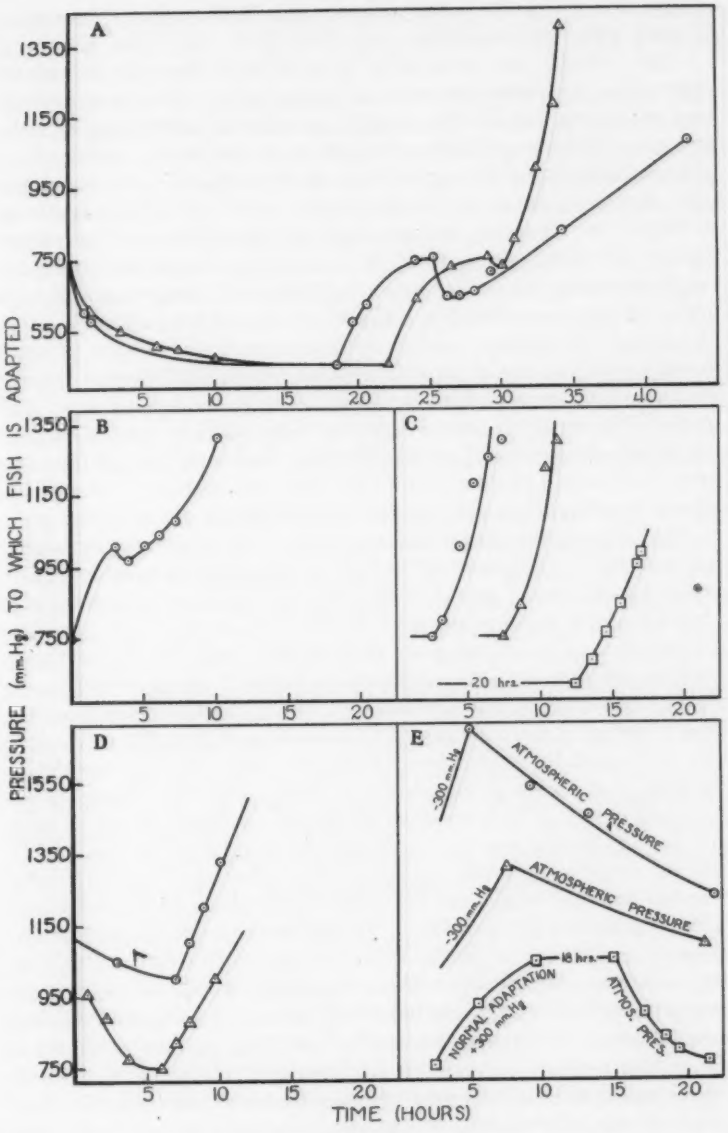
A
B
C
D
E
1350
1150
950
750
550
1550
5
10
15
20
25
30
35
40
20 hrs.
-300 mm. Hg
ATMOSPHERIC PRESSURE
18 hrs.
NORMAL ADAPTATION
+300 mm. Hg
ATMOS. PRES.
PRESSURE (mm. Hg) TO WHICH FISH IS ADAPTED
TIME (HOURS)

FIG. 3.

clistous fish, the perch, secretes into its swimbladder gas which consists of about eighty per cent CO_2.

These results may be applied to a physical interpretation of the observations that have been made upon the guppy. One would merely need to assume that the fish usually possesses in its bladder a partial pressure of CO_2 several times that in air at the water surface. The pressure decreases of the experiments (never more than about sixty per cent) would still favor the passage of CO_2 out of the bladder while one or both of the remaining two gases would diffuse inward. The magnitude of the partial pressure of CO_2 inside and the pressure decrease would determine the rate of outward diffusion of this gas and this rate might even be great enough to make the volume of the bladder decrease. Meanwhile, the response of the secretory mechanism to the pressure decrease would be one of minimal activity. As the CO_2 became depleted the rate of its outward diffusion would decrease correspondingly. The inward diffusion of the gases other than CO_2 would also tend to decrease the partial pressure of CO_2 in the bladder. Eventually, the partial pressures of all three gases would attain the same ratio as that of atmospheric air and then all gases would diffuse inward at rates to maintain this ratio.

Indirectly, some evidence has been obtained to support the hypothesis just described. We would expect that increasing the proportion of CO_2 in the bladder would permit a stronger opposition to the decrease in pressure, and a sufficient reduction in the bladder CO_2 would eliminate it altogether. The following are experiments which give such support.

Two male guppies were subjected to a pressure decrease of 300 mm. Hg in water brought into gaseous equilibrium at the reduced pressure. First there was a rapid increase in fish density and then after an hour or two a considerably slower one. These two rates were interpreted on the basis of the work of Meesters and Nagel (1934) to indicate an initial rapid escape of CO_2 and then the slower continued escape of other gases. The bladder was thus assumed to become relatively free of CO_2 and also to contain a smaller quantity of the other gases than originally. The fish was then restored to the original pressure in water equilibrated with air at the restored pressure. The gas secreted into the bladder was assumed on the basis of Jacobs' (1932) results to be richer in CO_2 than that which was withdrawn. When the proper volume of gas for the new pressure was reached and before the amount of CO_2 could decrease to equilibrium with a slower secretory rate, this fish was subjected to the sudden pressure decrease of 300 mm. Hg. This fish was able to oppose much more strongly the bloating associated with this decreased pressure than was the fish of the earlier experiments. Compare the results of experiments of this nature (Fig. 3*A*) with the results ob-

tained when guppies had neither appreciable gain nor loss in bladder gases over some days (Fig. 3*C*) but subjected to the same pressure decrease.

The same general results were obtained in a slightly different manner. A guppy was stimulated to decrease its density by subjecting it to an increase in pressure of 260 mm. Hg. At the moment of adaptation to the new pressure the fish was subjected to a pressure reduction of 560 mm. Hg (Fig. 3*B*). This was the equivalent of the usual reduction in pressure of 300 mm. Hg.

The reverse type of experiment was next performed. Guppies, for some hours adapted to increased pressures, were stimulated to let gases escape from their bladders. On the assumption that CO_2 is the gas to pass out most rapidly these fishes should, after considerable increase in density, have no larger percentage of CO_2 than is present in atmospheric air. And as expected the fish were at this moment unable to oppose in the slightest the bloating effect correlated with subjection to a sudden 300 mm. Hg pressure decrease. Figure 3*D* illustrates results of this nature.

A final test for the hypothesis was made to ascertain that the gas present in the fish bloating rapidly under decreased pressure did not contain as large a percentage of CO_2 as did the normally secreted gas. A guppy that had reached the maximum rate of bloating under the stimulus of the reduced pressure was suddenly restored to the original pressure. Under ordinary circumstances the initial response to such a decrease in pressure is a relatively rapid fish density increase which gradually or suddenly slows down. In the instance of this fish the initial response showed none of the usual initial rapidity and the whole process of return to the normal amount of gas for the pressure was relatively slow, as one would expect if the rate were determined by gases which were slow to diffuse (Fig. 3*E*). The normal rate of gas escape following secretion of gases into the bladder eighteen hours previously is also shown in the same figure for comparison.

Summary

1. A sudden and maintained pressure decrease from a value of one atmosphere to points between 625 and 300 mm. Hg results in an increase in gas content of guppy swimbladders and the rate of increase is an inverse function of the pressure.

2. This increase in gas content has been shown to be the result of lowering the swimbladder gas pressure, the gas tension of the body fluids then favoring passage of gases into the bladder.

3. Guppies are able to oppose more or less successfully the inward diffusion of gases at first, but gradually in the course of two to five hours such opposition ceases.

4. The transitory attempt on the part of the fish at density adjustment in response to the pressure decreases is explainable in the presence of an initially high partial pressure of CO_2 in the swimbladder.

5. The guppy appears to be unable to remove gases from the bladder when the diffusion gradient does not favor such passage.

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THE INTERNAL ANATOMY OF TWO PHALLOSTETHID FISHES

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INTRODUCTION

The Phallostethidae are highly specialized little fishes found in fresh and brackish water in certain limited regions of the Malay peninsula and the Philippines. They are of general interest in that they appear to provide a unique example of change of structure and function in a typical teleost organ, the pelvic fins of the male. The females are also modified, but to a lesser degree. The fishes derive their name from a large, muscular copulatory organ, the "priapium," suspended below the head in the male and which contains, in addition to the ductus deferens, the terminal apertures of the digestive and excretory systems (Figs. 1 and 2). In the female, the organ outlets are also anterior but, since there is no copulatory organ, the apertures are located on the ventral surface of the gular region (Fig. 3).

The phallostethids were first described and named by Regan in 1913 from a single species, but he notes a clear reference to their type by Duncker in 1904. Since Regan's work, twelve species have been discovered (Myers, 1928, 1937; Aurich, 1937). All are small, measuring from 14 to 40 mm. in length, and in 1935, after considerable study, Myers created for the group a new sub-order, Phallostethoidea, in the order Percesoces. Myers lists seven genera; Aurich groups them into three families and adds an eighth genus, *Solenophallus*.

Investigations on this group have been largely concerned with attempts to discover its taxonomic relationships, and a search has been made for possible homologies between the bony elements supporting the priapium and the bones of more typical teleosts.

Regan (1916), Bailey (1936) and Aurich (1937) have described the osteology of the priapium in several species of phallostethids. Bailey and Aurich each conclude that the priapial bones represent greatly modified pelvic fin and girdle elements, together with the first pair of ribs and, Bailey adds, possibly certain pterygials or rays of a portion of the pectoral fins. Aurich strengthens the argument that pelvic fin elements

have been transformed into priapial structures by describing the presence of vestigial fin rays in two species of the genus *Solenophallus,* and in *Gulaphallus mirabilis.* In all three species, he finds two or three delicate rays lying free in the skin behind the anus, and a second group of three rays inserted on the posterior extremity of the axial bone, a skeletal support which extends throughout the length of the priapium (see Aurich Fig. 3). In *Solenophallus,* these latter rays become comb-like at the ends. The females of several species possess a pair of papillae on either side of the oviduct opening (Regan, 1913; Herre, 1925, 1926; Villadolid and Manacop, 1934; Bailey, 1936). These may be vestiges of pelvic fins, and it is thought (Bailey) that the minute bony supports found in them in *Phenacostethus* may be remnants of the pelvic girdle. Villadolid and Manacop (1934) describe the development of the copulatory organ in *Gulaphallus,* showing it to arise from paired lobes surrounding the anus, on the ventral side of the throat. One of these lobes soon outgrows the other; the two fuse and gradually produce the elongate, asymmetrical priapium.

Brief descriptions of the soft parts of phallostethids have been published by Regan (1916) for *Phallostethus dunckeri* and *Neostethus lankesteri,* and by Villadolid and Manacop (1934) and Bailey (1936) for *Gulaphallus mirabilis.* There has been no detailed study of the gross and histological structure of the viscera, however, and it is to Dr. Hugh M. Smith that I owe the suggestion that such a study in these greatly modified forms would be of interest. Dr. Smith referred me to Dr. George S. Myers who supplied preserved material of two species, *Phenacostethus smithi,* and *Gulaphallus mirabilis,* from the collection at the U. S. National Museum. I wish also to acknowledge with appreciation the help Dr. Myers has given me in sending me a number of papers on the phallostethids.

Material and Methods

Phenacostethus smithi, described by Myers in 1928 from material collected by H. M. Smith in a fresh water stream in Siam, is the smallest phallostethid known (14–17 mm. total length) and is next to the minute Philippine goby, *Mistichthys luzonensis* (11–16 mm. total) in size. *Gulaphallus mirabilis* was described in 1925 by Herre as one of two species of a new genus which he had found in the fresh water streams of Luzon, the Philippine Islands. Its total length is not over 35 mm.

The specimens at my disposal were fixed in formalin and alcohol and preserved in alcohol. Transparent preparations of the skeleton were made by staining with alizarin and clearing in KOH and glycerine.

These were studied and dissected under a binocular dissecting microscope and other unstained specimens were dissected in alcohol for a study of organ arrangement. Sketches were made with the aid of a camera lucida. Serial sections were cut in transverse, sagittal, and frontal planes, 7–12 μ thick, and stained in iron haematoxylin, alum haematoxylin and triosin, and in Mallory's triple connective tissue stain. In all cases, fixation was poor for an adequate histological study and much more satisfactory results could be obtained with properly fixed material.

Skeleton

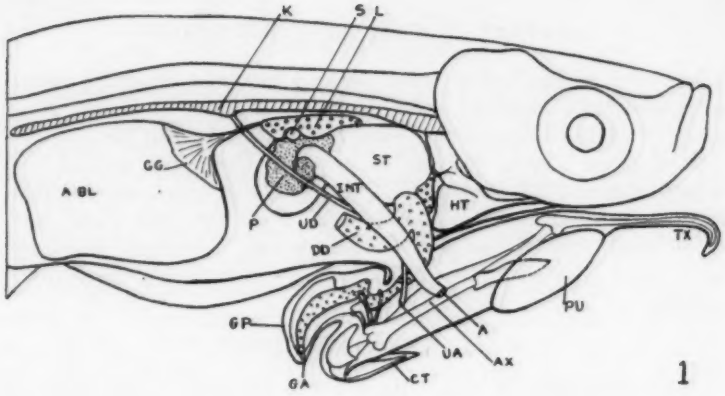
The descriptions of almost the entire skeleton of *P. smithi* and of *G. mirabilis* by Bailey (1936) and of the priapial skeleton of the latter species by Aurich (1937) give detailed information to which I can add nothing except to mention that Bailey did not notice the vestigial fin rays which Aurich finds in the priapium of *G. mirabilis*. After very careful examination, I have identified both the loose post-anal group of rays and the group articulated with the posterior end of the axial bone in that species, thus supporting Aurich (see Aurich, 1937, Figs. 3 and 8). In the priapium of *Phenacostethus*, however, I have found no trace of fin rays. This fish is much smaller than *Gulaphallus* and the skeletal elements are correspondingly more delicate, which may in part account for the disappearance of such minute vestiges.

Pseudobranch

The pseudobranch, which Aurich says is lacking in phallostethids examined by him, including *Gulaphallus*, is present in both that species and in *Phenacostethus* in the form of two minute tufts (Myers, 1928) lying in a recess at the anterior dorsal end of the sub-opercular cavity. Microscopic study shows this organ to belong to type II of Granel's (1927) classification of teleost pseudobranchs, in that the epithelium of the branchial chamber surrounds each tuft separately, but does not extend between the secondary lamellae of the tuft. Each primary lamella, or tuft, is furnished with an afferent artery and is supported by a precartilage rod which is extremely delicate in *Phenacostethus*. The secondary lamellae are close together and contain the large acidophile cells typical of the pseudobranch.

Viscera

The general arrangement of organs in *Phenacostethus* is shown in Figs. 1 and 2. Comparison with Regan's (1916) figures of *Phallo-*



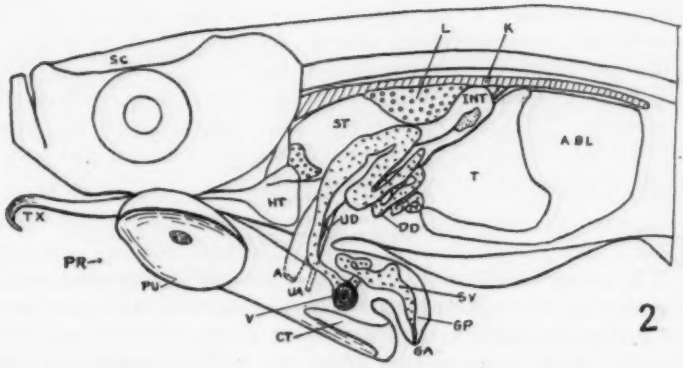


FIG. 1. *Phenacostethus smithi* ♂, internal organs in situ from the proctal side (× 16). Testis and portion of ductus deferens removed. The priapium is the large ventral appendage; several bones in it are outlined but Bailey's figures should be consulted for the complete skeleton.

FIG. 2. *Phenacosthethus* ♂, internal organs in situ from the aproctal side.

Abbreviations

A anus
ABL air bladder
AX axial bone
CT ctenactinium
DD ductus deferens
GA genital aperture
GG gas gland
GP genital papilla
HT heart
INT intestine
K kidney
L liver
PR priapium
PU sucker-like pulvinulus
S spleen
SC region of sensory canals
ST stomach
SV seminal vesicle
T testis
TX toxactinium
UA urinary aperture
UD urinary duct
V valve in ductus deferens

stethus dunckeri and with Villadolid and Manacop's (1934) figures of *Gulaphallus mirabilis* will give evidence of the great general similarity in the visceral anatomy of the group. Digestive and reproductive organs are located almost entirely in the space anterior to the air-bladder and, together with the excretory ducts, these systems terminate in apertures situated on the ventral surface of the throat region. It has already been pointed out (p. 59) that this extremely anterior position is associated with the male copulatory organ or priapium which is suspended from the head. The outlets are asymmetrically placed on the priapium; the anus and urinary duct open on one side, the proctal, while the ductus deferens ends in a genital papilla on the opposite or aproctal surface. As other workers have noted, this asymmetry is variable, that is, in some males the right side is proctal, in others, the left. In the female the apertures are mid-gular although no copulatory organ is present (Fig. 3).

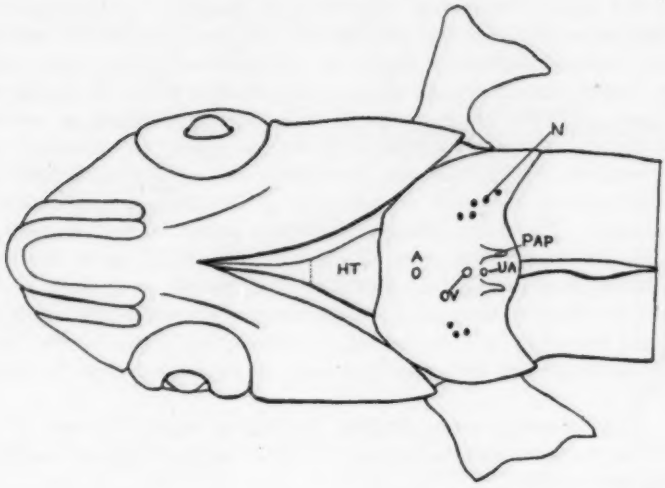
The following more detailed description applies to both *Phenacostethus smithi* and *Gulaphallus mirabilis* unless attention is called to one species only.

Endodermal Derivatives

The digestive tract is simple, short, and without pyloric caeca; a typical carnivorous type, as is borne out by Villadolid and Manacop in their analysis of the food of *Gulaphallus*. The intestine makes one complete coil in *Gulaphallus* and a partial coil in *Phenacostethus* (Fig. 1) before turning anteriorly toward the anus. The liver is large and lies close to the stomach and intestine (mainly on the aproctal side in the male); no gall bladder was identified. The pancreas is diffuse tissue, lying in the bend between stomach and intestine, with considerable fat scattered among its cells. Perhaps because of unsatisfactory fixation, no islet tissue is distinguishable. The spleen is partly embedded in the liver posterior to the stomach (Fig. 1). Thymus and thyroid glands are present. The large air bladder fills nearly one half of the body cavity. It lacks a pneumatic duct but is equipped in the anterior dorsal region with a vascular gas gland.

Nervous System

The size of the brain is large in proportion to the skull as in other minute fishes (TeWinkel, 1935). It was impossible because of poor fixation to determine whether the brain and cord are modified in relation to the priapium. Neuromasts are scattered on the surface of the body, particularly on the head region in *Gulaphallus;* a few are present in *Phenacostethus,* but here too, fixation was imperfect. Four large canals,



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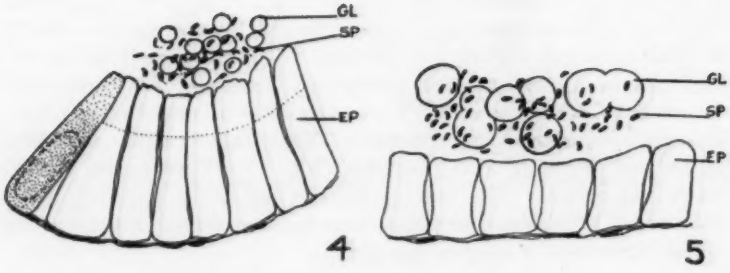

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FIG. 3. *Phenacostethus smithi* ♀, ventral view, anterior end (× 16). *A*, anus; *HT*, heart; *N*, neuromast; *O*, oviduct aperture; *PAP*, post anal papilla; *UA*, urinary aperture.

FIG. 4. Portion of proximal coil of ductus deferens in *P. smithi*. *Ep*, epithelium, showing in one cell probable position of nucleus; dotted line represents depth of lighter area in cells; *GL*, globules; *SP*, sperm head. Drawn with camera lucida under oil immersion lens.

FIG. 5. Portion of distal coil of ductus deferens from same section of *P. smithi* as Fig. 4. Epithelial cells are much lower and globules between sperm heads much larger than in the preceding figure. (Same magnification as above.)

with patches of sensory cells (which are probably lateral line organs) on their ventral inner walls, extend longitudinally on the snout and above the eyes in both species. These occupy considerable space between the skin and skull, and occasionally a homogeneous mass, possibly a cupula (Denny, 1937), is present in contact with the sense organ. The cavity containing the inner ear is proportionally large as in *Mistichthys*.

Kidney

The kidney in both species is a median organ which lies dorsal to the air bladder and digestive tract and diverges anteriorly into two sections which extend from below the occipital region of the skull up to the posterior level of the gills (Fig. 1). In *Phenacostethus*, and possibly in *Gulaphallus*, the urinary ducts are paired upon their exit from the kidney a short distance in front of the air bladder. They soon fuse, however, into a single duct which, in both species, passes between the intestine and gonad to open in the male on the priapium, directly behind the anus (Figs. 1, 2), and in the female, posterior to the oviduct in the gular region (Fig. 3).

Serial sections reveal one pair of pronephric glomeruli in both species. These lie dorsal to the heart and are directly attached to the dorsal aorta, which position is characteristic of the teleost pronephros. Poor fixation makes it impossible to tell whether the pronephric tubules are being replaced by lymphoid tissue, or whether they are functional, except the fact that the glomerular capillaries are full of blood cells, which suggests some function. The mesonephric glomeruli are smaller than the pronephric and are most numerous in that portion of the kidney anterior to the air bladder. The number of these glomeruli varies in *Phenacostethus* from 13 to 18, and in *Gulaphallus*, from 33 to 38, in the specimens where counts were made. In properly fixed material it would be more possible to determine, in so far as one could histologically, whether these fishes retain a functional pronephros in the adult, as is the case in *Fierasfer, Zoarces* (Emery, 1880), *Lepadogaster* (Guitel, 1906) and *Mistichthys* (TeWinkel, 1935). The number of glomeruli in *Phenacostethus* is only slightly greater than that in *Mistichthys* (which has 12 to 13 on the average) and it would be of interest to discover whether the ratio of glomerular volume to body surface is in any way comparable in these two unrelated minute teleosts.

Reproductive Organs

The ovary in the two species is single and lies along the anterior and ventral surface of the air bladder. In *Phenacostethus* there are on the

average fifteen maturing ova and the largest of these measure approximately 500 μ. Each ovum is surrounded by a thick porous zona radiata with surface filaments forming, but the method of filament attachment was not observed. The ovary of *Gulaphallus* contains about sixty maturing ova. The oviduct, in both species continuous with the ovary and doubtless formed by fusion of the mesovarium, is thick-walled and consists of a deep epithelial lining (possibly pseudo-stratified columnar) and a well-developed circular layer of smooth muscle. Its exterior opening lies just anterior to that of the urinary duct.

The breeding habits and development of *Gulaphallus* have been described by Villadolid and Manacop (1934). They demonstrate that the fish is oviparous but that fertilization is internal. No sperm are present in the oviducts of either species examined by me; the *Gulaphallus* material is immature and it is possible that *Phenacostethus* was collected at a time which was not the breeding season.

The testis is an unpaired organ, on the aproctal side partly wrapped around the anterior wall of the air bladder (Fig. 2). Seminiferous tubules open anterio-dorsally into a common collecting tubule. Because the structure of the ductus deferens differs considerably in the two species, each will be described separately.

In *Phenacostethus* all stages of spermatogenesis appear to be present in the testis. The ductus deferens is a long coiled tube, probably similar to that in *Phallostethus dunckeri* (Regan, 1913, 1916), forming a mass nearly equal to the testis in size. The tube is narrow in diameter for two or three coils, then increases to double its initial size, and finally extends into the priapium and into the genital papilla (Fig. 2). The epithelial lining of the tube is simple columnar, with tall cells in the narrower coils (Fig. 4), and cells about one-half this height in the large distal coils (Fig. 5), while that portion of the ductus deferens extending into the priapium is lined by low columnar epithelium. A valvular mechanism, greatly decreasing the lumen and lined by simple columnar epithelium, is present in the duct soon after it enters the genital papilla (Fig. 2). From this point the tube is slightly coiled, then dilates to form a seminal vesicle. Toward the tip of the papilla, the lumen is completely constricted and the wall seems to be composed of concentric layers of connective tissue, and very likely, some smooth muscle is also present.

The ductus deferens is crowded with spermatozoa in all specimens of *Phenacostethus* studied. In the narrow proximal coils of the tube (Fig. 4), there appear among the sperms small homogeneous globules which are, in all probability, secreted by the tall columnar epithelial cells of the lining. The size of these globules increases by one-half by

the time the large distal coils are reached (Fig. 5) and this increase may be the result of fusion of smaller globules. The staining capacity of these bodies varies: in Mallory's triple connective tissue stain, they are purplish in the proximal coils, but distally, brilliant red; in iron haematoxylin, brownish proximally, and distally gray or black; in alum haematoxylin and triosin, uniformly pink. These globules may be gelatinous spermatophores, but there seems to be no definite association between them and the sperms such as is described by Regan (1916) in two other phallostethids. Some spermatozoa may adhere to them, but there are quantities of free sperm in all parts of the duct. There is also a possibility that globules and sperm become united upon discharge, but study of living material is necessary to determine it.

The testis in the male specimen of *Gulaphallus* which was sectioned is large but immature.. The ductus deferens, within the body cavity a very narrow duct probably without secretory function, dilates in the posterior portion of the priapium to form a seminal vesicle. The duct is of course entirely devoid of sperm so that the presence of sperm packets, affirmed by Bailey who evidently studied more mature specimens of this species, was not observed. (See Pl. 3, fig. 3 in Villadolid and Manacop, 1934).

Study of the two phallostethids described in the preceding pages, in spite of the handicap of poorly fixed material, has shown that, histologically, there are no striking differences in the viscera that may be attributed to the priapium or to the displacement of the organ outlets to the throat region. The persistence of the pronephros, which may be functional in the adults, is of general interest because it occurs so rarely in teleosts; it would be of value to examine other members of the group to determine whether that organ is always present in phallostethids. The great difference in the structure of the ductus deferens in the two species is undoubtedly related to family specialization. Possibly, the adult male *Gulaphallus* develops a glandular groove externally (as in *N. lankesteri,* Regan, 1916) to compensate for lack of abundant secretion on the part of the sperm duct; such a groove was not seen, however, in the pre-adult male examined histologically.

Summary

The anatomy of the soft parts of two phallostethid fishes, *Phenacostethus smithi* and *Gulaphallus mirabilis,* has been studied.

1. Two groups of vestigial fin rays are present on the copulatory organs of *Gulaphallus* but are lacking on that of *Phenacostethus.*

2. A tuft-like pseudobranch, belonging to Granel's type II, is present in both species.

3. The viscera are located almost entirely anterior to the air bladder, and digestive, reproductive and excretory systems open externally on the sub-gular copulatory organ in the male and on the ventral surface of the gular region in the female. Liver, pancreas, spleen, thymus and thyroid are identified.

4. Neuromasts are scattered on the surface, and four canals, probably of the lateral line type, are present on the heads of both species.

5. The kidney contains one pair of pronephric glomeruli which may be functional. There are 13–18 mesonephric glomeruli in *Phenacostethus*, and 33–38 in *Gulaphallus*.

6. The ovary is single in both species and contains a relatively small number of ova. The oviduct, continuous with the ovary, is thick-walled and muscular.

7. The testis is a single mass. Seminiferous tubules open into a collecting tubule which continues as the ductus deferens. This duct forms a large coiled mass in *Phenacostethus* and is lined by simple columnar epithelium. Homogeneous globules, present among the sperms in the lumen, do not appear to form spermatophores. The ductus deferens in *Gulaphallus* is very narrow within the body cavity, but dilates to form a seminal vesicle in the copulatory organ.

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REGENERATION OF GONAD TUBULES FOLLOWING EXTIRPATION IN THE SEA-CUCUMBER, THYONE BRIAREUS (LESUEUR)

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Several accounts infer that the extensive powers of visceral regeneration in the holothurians may extend to the gonads (Torelle, 1909; Deichmann, 1921; Bertolini, 1932) although they give neither precise data regarding the portion of the gonad involved nor even a gross analysis of the regenerated tissues. The present study considers the general problem of gonad regeneration in the holothurians with special reference to three specific points, namely, (1) the extent of the capacity to regenerate gonadal tissue, (2) the presence or absence of germ cells within regenerated tubules and (3) the possible origin of any germ cells found within them.

Materials and Methods

Previous experience demonstrated (Kille, 1931) that *Thyone briareus* (Lesueur) is an extraordinarily good holothurian for experimental purposes. In contrast to the genus *Holothuria* which eviscerates through a tear in the cloaca, this genus casts off the entire anterior end of the body whenever it autotomizes the digestive system. With these parts lost, the animal is nothing but a dermo-muscular sac containing only a cloaca, respiratory trees, gonads, and mesentery. If left to itself, the circular body muscles at the extreme anterior end contract strongly so as to close off the body cavity from the sea. A healing process soon makes this closure permanent. However, if one makes a longitudinal cut at the anterior end equivalent to about one-fifth the length of the animal, many specimens may be turned inside out following their evisceration. In such a position the gonad tubules and the mesentery hang freely in the water. (Fig. 1, *A, B, C*). With the animal in this position, operations on the gonad are possible. Most specimens can then be turned right side out with little difficulty. If the animal is returned to running water, a high percentage survive the operation (Kille, 1937). In some cases the circular muscles of the body wall contract so

strongly that the animal cannot be turned right side out. Such individuals do not live more than a day or two.

The Reproductive System in Thyone briareus (Lesueur)

A thick tuft of gonad tubules is found in each side of the dorsal mesentery as seen in Fig. 2. They originate from tissue localized within the mesentery in a region near the dorsal body wall (Fig. 2, gonad-basis) and about midway between the mouth and the anus (Fig. 1, *C*). These tubules empty their products into a common chamber located within the dorsal mesentery (gonad-basis chamber, Fig. 2). Anterior to the tubules this cavity continues as the lumen of a gonoduct which runs through the dorsal mesentery to open exteriorly at a point between the bases of the two dorsal tentacles. Each right and left half of the

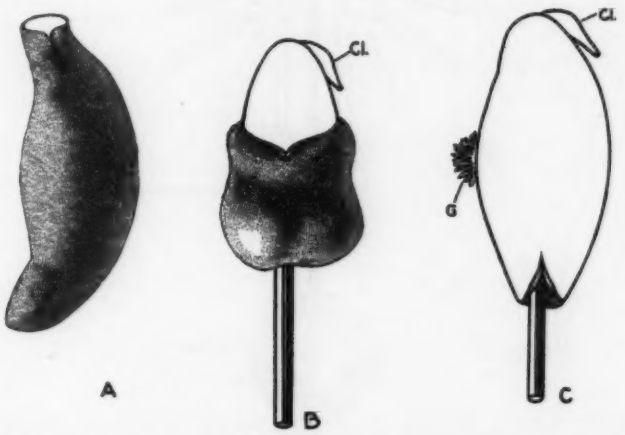

Fig. 1. *A, Thyone* immediately after evisceration with a short longitudinal slit through the body wall at the anterior end (ventral surface to the right); *B, Thyone* being turned inside out with the aid of a blunt glass rod; *C*, specimen turned completely inside out. *Cl, cloaca;* G, gonad. (Mesenteries and musculature of body-wall omitted.)

Thyone gonad consists then of (1) a lateral portion of a chamber located within the dorsal mesentery (the gonad-basis) and (2) a mass of tubules which take their origin from the ventro-lateral wall of the chamber (gonad tubule, Fig. 2). The right and left tufts of tubules are symmetrically developed, there being but little variation in the number and size of tubules between the two sides. Within each tuft, however, one can distinguish tubules in various stages of development. The ex-

treme anterior ones are small, unpigmented, translucent, immature tubules. Such a condition has been described for many genera (Théel, 1901; Mitsukuri, 1903; Haanen, 1914; Deichmann, 1930, etc.). Posterior to these is a group of tubules which grade into the slender, more mature tubules possessing pigmented, opaque walls, rounded extremities and greater length. These long, opaque tubules make up the bulk of the gonad, yet the shorter, anterior tubules comprise at least a fifth of the total number of tubules present. It should also be mentioned that

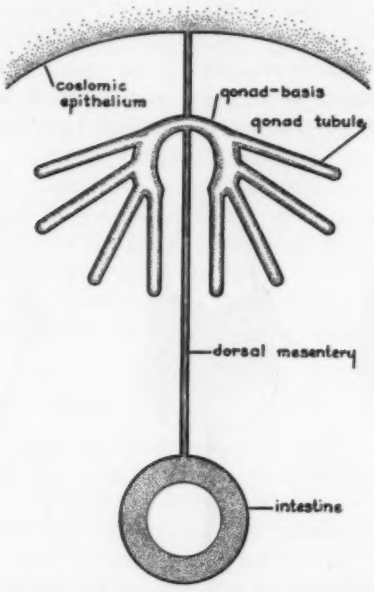

FIG. 2. Diagrammatic representation of a cross-section through a piece of the dorsal body wall with the attached mesentery to show the relationship existing between body wall, mesentery, gonad and intestine.

in the larger specimens collected during July and August there is an indication that posterior to the tuft of tubules still older tubules may have been lost.

In the medium-sized specimens used in these experiments, one can say that the larger the specimen, the greater is the mass of the gonad for the tubules are not only larger but their number is greater (see Table I). Within two larger specimens whose body volume was approximately 140 cc., the count for the female was 400 tubules while the male possessed 1035.

All these data indicate that there is a continued anterior proliferation of the tubules from the gonad-basis. Histological analysis of the tubules supports this conclusion. The posterior tubules show various stages in gametogenesis though there are fewer mature germ cells present than in those tubules more anterior, which may be packed with well-developed gametes. The most anterior tubules, however, show no ripe gametes but enormous numbers of germ cells in the early stages of gametogenesis. At the present time, I am not prepared to give an account of the seasonal variation in the gonad for all material discussed in this paper, whether normal or experimental, was collected in the months of July through October.

TABLE I

The total number of tubules found within a series of eleven specimens closely graded as to size.

Size of Specimens (Volume in cc.)	Approximate Number Tubules in Each Tuft ♀	♂
4	130	
4.5	110	
5.8	107	200
6.6	150	
8.0	160	
10.0		250
12.0	185	
13.0	170	
14.0	200	
19.0	170	

RESULTS OF THE REMOVAL OF THE GONAD TUBULES EITHER WITH OR WITHOUT THE GONAD-BASIS

Since it is possible that part of the gonad-basis is torn away with the gonad tubules whenever they are entangled in the digestive system during the process of evisceration, two types of operations were carried out. In Group I, the gonad tubules and the entire gonad-basis were extirpated from 6 female and 6 male specimens of medium-size. These were collected at Woods Hole in early August. In Group II, composed of a similar group of specimens, the gonad tubules were torn away from the gonad-basis by scraping the gonad area with the blunt back of a scalpel. This was done in order to produce a condition similar to that which occasionally exists after tubules have become entangled in a digestive system that is being eviscerated. Though this seldom happens in *Thyone briareus* (Lesueur) at this season, it frequently occurs in certain Holothuria when the gonads are well-developed.

In both groups, the ability to feed indicated that the new digestive system was established within 15 to 22 days, which is the average period of time required for unoperated specimens (Kille, 1935) as well as for the controls to these experiments. Evidently this unusual procedure for a coelomic operation has no injurious effect. None of Group I, however, showed any signs of gonad regeneration. Eleven specimens were killed for examination at various intervals after the operation, ranging from 46 to 129 days. The other remaining specimen of the

FIG. 3. Ventral view of the testes within a *Thyone* 63 days after the main mass of the gonad had been scraped away. For size comparison, the proximal quarters of 5 mature tubules of the original right testis are included on the reader's left.

group was in poor condition at the end of the experiment and was discarded. In all cases the new digestive system and its associated structures were well developed but the cut end of the gonoduct had healed blindly.

On the other hand, the gonad-basis of each specimen in Group II proliferated either a right, or a left, or a right and a left tuft of miniature tubules. Evidently all of the very smallest tubules of the original group were not always rubbed off for some of the newly proliferated groups showed a few tubules so much larger and longer than the rest

that it was clear that these belonged to the original gonad mass. Such groups were thus composed of a large number of newly proliferated tubules and, in addition, a smaller number of tubules which were present before the operation (Fig. 3, left gonad). This assumption is supported by the microscopic examination of a number of specimens which were killed immediately after the operation. In some cases, one could identify very minute tubules which remained attached to the remnant of the gonad-basis. At least this remnant must certainly remain even after the most violent self-eviscerations of holothurians.

The method of operation, however, frequently resulted in the removal of all the original tubules. Such an operation would easily account for those newly regenerated tufts which are like the right testis in Fig. 3. All the regenerated tubules are small and equal in development. This condition stands in sharp contrast to what is found in a normal male of equivalent size and season. Typically there would be only a fourth of this number of minute tubules and they would show a graded series as to length. Further evidence that these tubules arose at the same time is furnished by their histological similarities. In each tubule, the germ cells are packed rather solidly within the tubule. In contrast to this, the longer tubules as seen in the left testis (Fig. 3) possess a germinal epithelium surrounding a definite central lumen.

The Origin of the Germ Cells within the Newly Proliferated Tubules

Since the microscopic examination of the newly proliferated tubules showed that they were packed with cells which were identical cytologically with the primordial germ cells found within the tubules of the normal gonad, a careful study was made to determine the origin of these cells. Sections were prepared of normal gonads and of the gonad remnants left within operated but unregenerated specimens. Special attention was given to the anterior region of the normal gonad where the immature tubules occur. Here the small, club-shaped tubules appear to be diverticula of the wall of the gonad-basis. Anterior to these, one finds a few, minute, knobbed protuberances of the wall, each of which possesses a solid core of germ cells. Apparently, each core originates from an independent center within the wall of the gonad-basis, for just anterior to the protuberances separate nests of germ cells are found in those tissues. In view of their size and position, it is not surprising that they have never been described in this species. These nests of germ cells are evidently the equivalent of the "genital stolon" which is quite conspicuous in the dorsal mesentery of some holothurians (Théel, 1901;

MacBride, 1906) and from which the gonad-basis, gonad tubules and germ cells arise.

Examination of remnants of the gonad-basis left within operated animals shows that nests of germ cells may or may not be left on either side as a result of the operation performed upon Group II. This would seem to correlate with the fact that new tubules packed with germ cells may be proliferated on either or both sides following such operations. It is assumed then that such residual nests of germ cells are the source of the germ cells found within the newly proliferated tubules. Though none of these 12 animals failed to proliferate tubules on at least one side, one would expect this to occur occasionally if this assumption is true.

Discussion

Since only a small part of the gonad-basis is ever lost when the gonad tubules are torn out during a process of visceral autotomy, these results show that an eviscerated holothurian does not merely mature certain small, residual tubules but may actually proliferate an entirely new set of gonad tubules. The removal of the original set of tubules is, in reality, a removal of only that part of the gonad in which a portion of the germ cells are maturing. The remnant of the gonad-basis possesses a store of primordial sex cells which are then contributed to the new tubules as they are proliferated at a rate probably four times the normal. It is a case similar to that reported in vertebrates where growth of accessory or residual gonadal tissue may follow incomplete gonadectomy. It is apparent, therefore, that true "regeneration of the gonad" does not occur but merely a proliferation of tubules from a growth zone or area of the gonad-basis.

In a period of 4 months, only a capacity for healing is demonstrated by the tissues of the gonoduct or of the gonad-basis when germ cells are absent. That the proliferation of tubules by the gonad-basis does not occur in the absence of germ cells is suggested by the facts that (1) a tubule stump (which contains no germ cells) never produces a new tubule, and (2) among the new proliferations, no sterile tubules have ever been found. It will also be noted in Fig. 3 that the tubules take their origin from an extremely localized area of the gonad-basis. This manner of origin suggests a dependence upon a restricted group of cells rather than upon a general capacity of cells widely distributed throughout the gonad-basis. It is of interest in this connection to note that in the chicken, Willier (1937) has obtained by means of chorio-allantoic grafting, male-like sex cords even though no primordial germ cells are present. A sexual gland without germ cells may also develop

in the urodele under certain conditions (Humphrey, 1928). Furthermore, Geigy (1931) has shown that in *Drosophila* the formation of a gonad is not dependent upon the presence of germ cells. By means of ultra-violet radiation he destroyed the primitive sex cells in the egg yet he obtained sterile ovaries and testes.

This dependent relationship of the origin of tubules upon the presence of germ cells in *Thyone* may be more apparent than real. A small group of cells at the anterior limit of the gonad area may retain a capacity for tubule formation whereas cells at more posterior levels do not. The relation then might be one of position only, the removal of the germ cells which are in this region invariably involving a loss of these tubule-forming cells. Microscopically there is no evidence for such a differential capacity among the cells of the gonad-basis. The only striking histological difference along its antero-posterior axis is the presence or absence of germ cells.

These results raise an interesting question relative to those holothurians such as *H. parvula* which undergo transverse fission in nature. In these species the gonad is located far anterior and therefore it is retained by the anterior portion. If the regenerative capacity of this genus is as limited as that of *Thyone,* then the posterior portions must continue their reproduction entirely by asexual means. While the occurrence of fission has frequently been reported in certain species of holothurians (Crozier, 1917; Deichmann, 1921; Kille, 1936), there has been no demonstration that it is the sole means of reproduction for certain individuals sterilized by the plane of a previous fission. Deichmann has reported (1921) "feebly developed" gonads within regenerating portions of *Holothuria* (*Actinopyga*) *difficilis.* In a recent communication to the author this investigator states that these gonads were seen only within anterior portions. There is then no report in the literature that the posterior portions ever develop a gonad. The absence of any such natural evidence for the regeneration of the gonads from other tissues coupled with the data obtained through this direct experimental approach makes a strong case for the segregation of tissues with germinal potencies within a highly restricted region of the dorsal mesentery.

As Stolte has recently (1936) pointed out, little is known concerning the cellular basis of regeneration within the echinoderm. Zirpolo (1928) believes there is a widespread totipotency among cells throughout the body of *Asterias.* On the other hand, studies on the genera *Stichopus, Holothuria* and *Thyone* leave us with only one established case to support such a generalization for Holothuria, namely, the formation of a new gut epithelium from the cells of the mesentery in *Stichopus regalis* (Bertolini, 1930). In all other instances new tissues apparently

arise from remnants of the old with the one possible exception of the anterior gut epithelium within a regenerating *Thyone* (Kille, 1935, p. 93). Since holothurians show an unusual capacity for regeneration there has been a tendency to assume that the tissues (or a tissue) of this group possess a totipotency throughout the entire animal. In light of the evidence, this assumption is unwarranted. Apart from the problem of organization, the unusual restorative powers are simply evidence of a normal capacity for cellular proliferation or migration and the maintenance of differentiation under new conditions.

Summary

(1) The gonad in the sea-cucumber consists of two major portions: (*a*) a group of tubules in which germ cells are maturing and (*b*) a basal portion, the gonad-basis, from which these tubules arise. Failure to recognize this has probably led to the incorrect statements that these forms "will regenerate the gonad."

(2) In order to test the extent of the capacity to regenerate gonadal tissue, a partial or a complete gonadectomy was performed upon *Thyone briareus* (Lesueur). The gonad was exposed by turning the animal inside out after an induced autotomy of its digestive system and anterior end.

(3) That this procedure had no very injurious effects is shown by the fact that approximately 95 per cent of the operated animals survived. These and the controls regenerated the autotomized parts as rapidly as did eviscerated but unoperated animals.

(4) There was no regeneration of gonadal tissue following complete gonadectomy.

(5) If only the tubules were removed, new tubules might arise from a restricted, anterior region of the gonad-basis at a rate four times the normal (at this season).

(6) The only histological feature that distinguished this productive region from the rest of the gonad-basis was the presence of nests of germ cells.

(7) The possibility that tubule formation is dependent upon the presence of germ cells was suggested by still other facts. No sterile tubules were ever found among the regenerated tubules. Furthermore, the sterile proximal portion of a tubule which sometimes remained after a tubule had been torn out, did not give rise to a new tubule. Finally, when the tubules were roughly torn out, that portion of the gonad-basis containing the germ cells might or might not be lost and this is correlated with the fact that after such treatment, a *Thyone* may regenerate either a right or a left, or a right and a left tuft of tubules. It is

granted, however, that such evidence is not conclusive. Germ cell origin and tubule formation may be independently related to a totipotence possessed by only a few cells in a restricted portion of the gonad-basis for which these experiments provide no test.

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EGG LAYING IN THE ACOELOUS TURBELLARIAN POLYCHOERUS CARMELENSIS[1]

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The few accounts of egg laying in the Turbellaria Acoela are brief and unsatisfactory. The Acoela have one or two genital pores, except Nemertoderma (Steinböck, 1931). The absence of oviducts in the Acoela (Bresslau, 1933) leaves the question of egg emergence unsolved.

Von Graff (1905) concluded that the eggs of *Otocelis rubropunctata, Polychoerus caudatus* and *Convoluta roscoffensis* are liberated through the genital pore, which he considered the normal method for most Acoela. For species which possess no oviducts he agreed with Weldon, Sabussow and Monticelli that the mouth might serve in egg deposition. Yet von Graff pointed out (p. 1960) that oviducts had been described with reasonable certainty for only *Otocelis rubropunctata* and *Polychoerus caudatus* and that *Amphiscolops langerhansi* possesses no formed passage.

The evidence supporting von Graff's conclusions as to the rôle of oviducts and genital aperture was extremely meager. In his account of egg deposition in *Otocelis rubropunctata* (1904) he does not mention the path of emergence of the eggs. Gamble and Keeble (1903) stated that in *Convoluta roscoffensis* " frequently the body breaks in two across the opening of the oviduct . . . ," i.e., presumably at the level of the genital pore. Gardiner (1895) is the only observer who has definitely stated that he witnessed the emergence of the eggs from the genital pore. He undoubtedly considered that the eggs of *Polychoerus caudatus* reach the female pore through the oviducts described and figured by Verrill (1893) and later by himself (1898). In these accounts the term oviduct was substituted for vitellarium, and the vitellaria were figured as uniting into a common duct leading directly to the female aperture. Mark (1892) found no connection between the vitellaria and the genital pore, and Löhner (1910) showed conclusively that this species possesses no oviducts. Löhner considered it probable that the eggs pass through a series of vacuoles in the marginal parenchyma and out by way of the female aperture.

[1] This investigation was aided by a grant from the Society of Sigma Xi. The authors are indebted to Dr. C. E. McClung for reading the manuscript.

Subsequent references to egg emergence in the Acoela are equally inconclusive. Von Graff (1911) concluded with reference to *Anaperus gardineri* that the eggs probably leave by way of the mouth or through breaks in the body wall. With regard to the entire group Bresslau (1933, p. 118) stated that the eggs probably pass through "einfache Gewebslücken in das Zentralparenchym, um dann durch die Mundöffnung nach aussen befördert zu werden. Gelengentlich vollzieht sich ihr Austritt noch direkter; durch Ruptur der Körperwand an irgendeiner Stelle." This latter alternative obtains in the case of *Polychoerus carmelensis.*

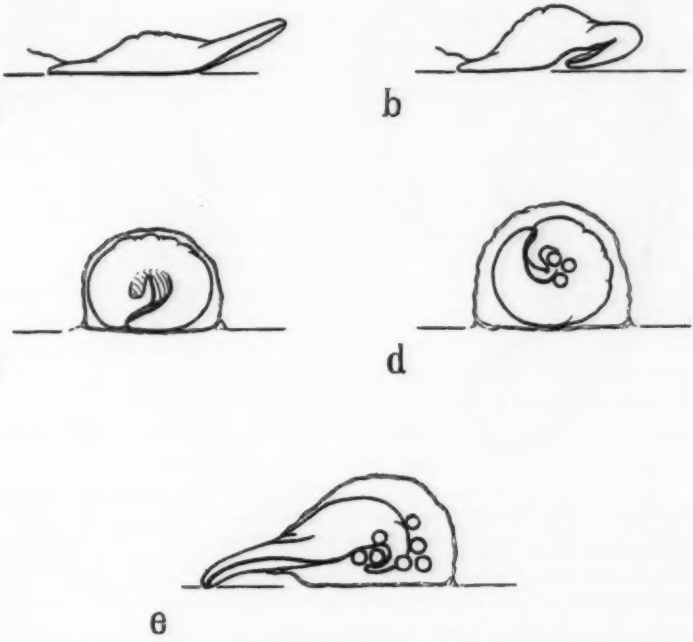


FIG. 1, *a-e.* Series of sketches to illustrate progressive stages of the process of egg laying.

MATERIAL AND METHODS

The reproductive system of *Polychoerus carmelensis* has been described and figured in a recent paper (Costello and Costello, 1938). Specimens about to deposit eggs were observed in situ by means of a hand lens or binocular microscope. Removal for examination invariably terminated the process. Twelve specimens were fixed during egg

laying, as follows: five in hot Heath's solution (50° to 60° C.), three in Lillie's, two in Worcester's (one "warm," one at 50° C.), one in Flemming's and one in Champy's solution. The subsequent study of hundreds of specimens has shown that hot fixatives are best for the general preservation of tissues. The specimens were sectioned individually and serially.

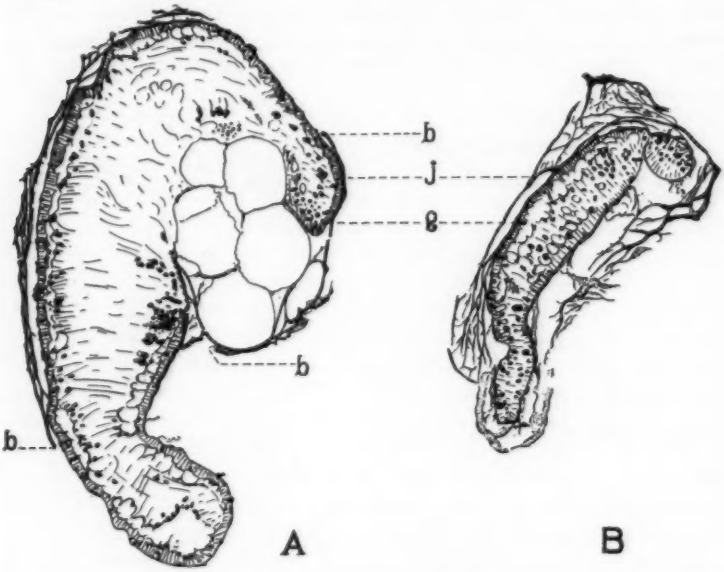


FIG. 2. Oblique sections of an egg-laying individual preserved in Lillie's fixative and stained by the Flemming tricolor method. *A*, through the side of the body; *B*, near the anterior end. To show jelly layers (*j*) of the egg mass, surrounding the worm, and the glands (*g*) which secrete the jelly. In *A*, the jelly is broken at several points (*b*); in *B*, the jelly mass is more complete. Magnification 43 ×.

OBSERVATIONS

An individual about to deposit eggs assumes a resting position on the substratum. The anterior end of the body is at first elevated (Fig. 1*a*), as the animal sways slowly from side to side, and is then bent ventrally in progressive stages (Fig. 1*b*). Meanwhile a jelly-like slime is secreted from the surface of the body and adheres to the substratum. As the anterior portion of the body continues the ventral curling, the posterior end relinquishes its attachment, and curls ventrally under the approaching anterior tip, forming a ball which is attached to the sub-

stratum only by the secreted jelly (Fig. 1*c*). During the curling, and subsequently, violent contractions are observable in the region of the vitellaria. The animal then begins to rotate, end over end, rather slowly within the gelatinous mass. During rotation, additional jelly is secreted by the surface of the animal (Fig. 2). Meanwhile, the eggs are emerging from the body of the animal, but are, for the most part, hidden within its folds (Fig. 1*d*). An occasional egg escapes laterally, and may then pass freely over the dorsal surface of the rotating animal (Fig. 5), within the jelly. Rotation continues for three or four minutes, after which the animal unrolls and works its way out of the jelly mass (Fig. 1*e*) through an opening so small as to greatly constrict the body. The eggs are left behind in the central cavity of the jelly mass, which contracts and forces the eggs into close proximity. The entire process of egg laying requires only five or six minutes.

Egg laying occurs infrequently during the early portion of the day, most frequently shortly after sunset (Table I). That egg laying occurs also during the early night is evidenced by large numbers of egg masses found in the morning in late cleavage stages.

The point of emergence of the eggs is concealed by the position assumed by the body during the process. Living individuals forcibly unfolded during egg emergence usually extruded no more eggs. In one case, however, an egg was observed to emerge through the ventral body wall at the level of the bursa.

The fixed material showed very clearly the position occupied by the mature eggs immediately before extrusion, and the point at which the eggs are shed, although no individual egg was fixed in the act of emerging. Of the twelve specimens, one had shed all its mature eggs, three had shed none, the others, a portion.

It has been noted (1938) that the oöcytes move dorsally within the vitellaria as they mature. However, eggs undergoing maturation occupy the greater part of the dorso-ventral thickness of the vitellarium. Immediately before deposition the mature eggs are located medially and ventrally between the body wall and the ventral surface of the bursa. The vitellaria are displaced laterally and the bursa is displaced dorsally, curving around the eggs as shown in Fig. 3. This arrangement was evidenced by the eleven fixed specimens which retained one or more mature eggs.

Evidence as to the point of emergence of the eggs is limited to the nine specimens which had shed one or more eggs at the time of fixation. There was no indication of the emergence of eggs by way of the mouth and intestinal parenchyma, nor by way of the vagina. Three of the five best preserved specimens showed perfectly definite points of rup-

TABLE I

The numbers of egg depositions observed in the laboratory are listed according to the dates and hours of occurrence. The hours of observation (—) on the respective dates are indicated.

Date	A.M.							P.M.												Total
	5–6	6–7	7–8	8–9	9–10	10–11	11–12	12–1	1–2	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10	10–11	11–12	
8/9	—	—	—	—	—					—	—	—	—	—	—	—	2			2
10						—	—	—						—	4	3	1	—		8
11						—	—	—						1	—	—	—	—	—	1
12						—	—	—		—	—	—	2			—	—	—	—	2
17						—	—	—		1	—			—	—					1
19						1	—			—	—				—	—				1
23						1	—			—	—				1					2
24	—	—	—						—	—	—	—	1							1
26						—	—	—	—	—	2	—								2
27							—	—		—	—	1								1
Total						2				1	2	1	3	1	5	3	3			21

ture of the ventral integument in the region of the bursa, with the eggs in such a position as to indicate their emergence at this point.

The best two cases are illustrated by Figs. 4 and 5. In Fig. 4, the section passes through the flattened vagina (*v*), the female aperture (♀), the ventral protuberance in which two of the mature eggs appear, and

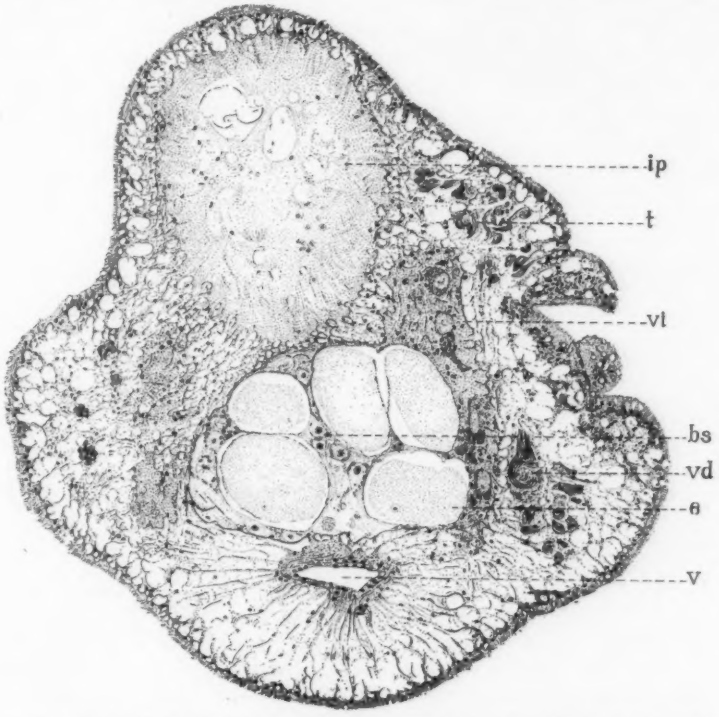
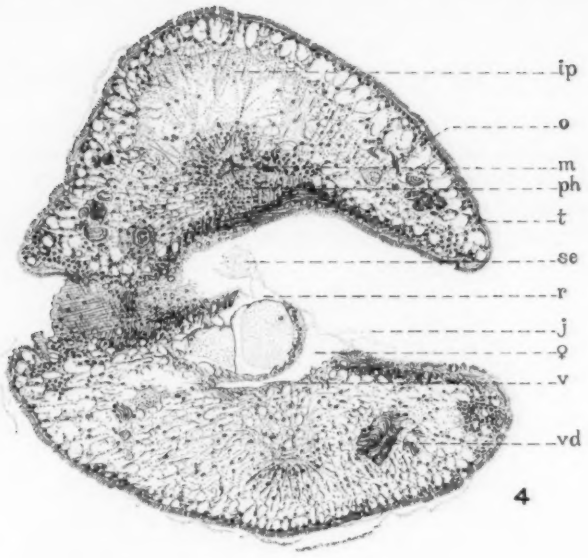


FIG. 3. Oblique frontal section of an egg-laying individual, to show the median ventral position of the mature eggs (*e*), and the displacement of the bursa (*bs*) around the eggs. Hot Heath fixation, stained in Heidenhain's haematoxylin. Magnification 72 ×. *ip*, intestinal parenchyma; *t*, testis follicles; *v*, vagina; *vd*, vas deferens; *vi*, vitellarium.

through the undisturbed mouth (*m*) and short pharynx (*ph*). The ventral integument shows a rupture (*r*) about 50 microns in width near one of the shed eggs (*se*). It is medial, extending through seven consecutive sections (70 microns). A jelly layer (*j*) surrounds the shed eggs and is connected with the ventral surface of the body near the point of rupture. The unshed mature eggs converge toward the point of



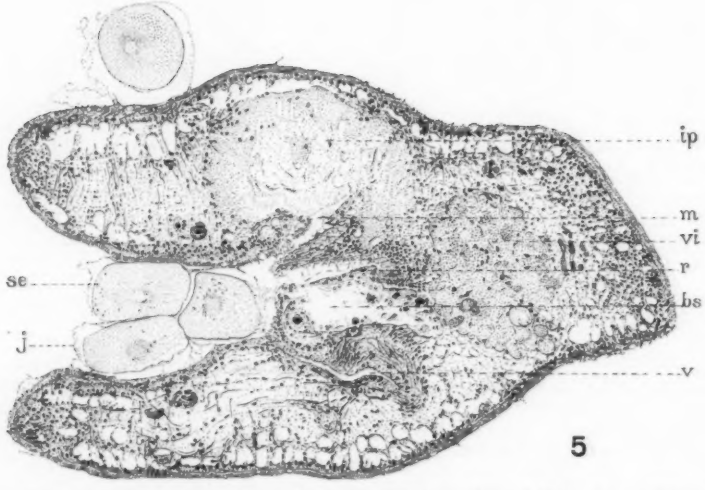


Fig. 4. Same individual as Fig. 3; twentieth section (0.20 mm.) ventral to that of Fig. 3. To show the rupture of the ventral integument (*r*), and the jelly strand connecting one of the two already shed eggs (*se*) with this point. The section passes obliquely frontally through the mid-region and nearly transversely through the mouth (*m*) and pharynx (*ph*). Magnification 50 ×. ♀, female genital aperture; *o*, immature egg; other abbreviations as above.

Fig. 5. Oblique sagittal section of an individual fixed after the extrusion of all of its eggs. To show the broken ventral integument and disrupted bursa, also the undisturbed mouth and intestinal parenchyma, and the flattened vagina. Hot Heath fixation, stained in Heidenhain's haematoxylin. Magnification 62 ×. Abbreviations as above.

rupture, as shown by the sections of the same individual represented in Figs. 3 and 4. The second specimen, represented in Fig. 5, shows the relation of the bursa, ruptured integument and shed eggs. In this case also the condition of the mouth and the extremely flattened vagina present striking evidence that these structures are not concerned in egg deposition. The ventral integument in the region of the bursa is disturbed and lacks cilia. Bits of tissue and jelly extend from the last shed egg to the region of the rupture. The bursa is greatly vacuolated, a portion of its tissue extending to, and apparently partially closing, the rupture. The disruption of the bursa results indubitably from the pressure of the eggs in their outward passage, combined with the violent muscular contractions observable in this region throughout the egg-laying process.

TABLE II

Distribution of the numbers of eggs per egg mass deposited in fingerbowls by animals collected 8/20/37, from the third to eighth day in the laboratory.

	Numbers of Eggs per Egg Mass					Total Numbers of Egg Masses
	1–5	6–10	11–15	16–20	21–25	
8/23	23	72	27	3	1	126
8/24	3	4	3	0	0	10
8/26*	14	34	19	2	0	69
8/27	3	13	19	5	0	40
8/28	5	18	12	2	0	37
Totals	48	141	80	12	1	282

* Two-day interval.

In the remaining seven specimens the exact point of rupture was not clear as a result of either the plane of sectioning, or of distortion following slow fixation. However, they correspond, in all essential features, with the two cases described above.

If *Polychoerus carmelensis* regularly discharges its eggs through rupture of the body wall, evidence of this should be observable in mature specimens fixed at random. Histological examination has shown frequent irregularities in the epidermis, parenchyma and bursa wall, including, in some cases, the interruption of epidermal ciliation. The general appearance suggests regeneration following recent disruption.

A few miscellaneous observations regarding the breeding habits of *Polychoerus carmelensis* in the laboratory and in the tide-pools may be here recorded. Animals brought into the laboratory and placed in fingerbowls do not, as a rule, begin to deposit eggs (or continue egg deposi-

tion) until the third day thereafter. During the following three days egg clusters are deposited in abundance, after which they become less numerous. The individual worms do not usually deposit all of their eggs at once, as has been suggested for the closely related *Polychoerus caudatus*. This is attested by the fact that the number of egg masses obtainable from a single dish may exceed the number of worms present. Data relating to the numbers of eggs per egg mass are given in Table II. The figures represent the numbers of egg masses deposited in five fingerbowls during the first eight days in the laboratory.

There appears to be a definite breeding season for this species in the Monterey region. Collections in 1937 were made at intervals from June 22 through July and August. In June no egg masses or young worms were found in the tide pools. By July 8 they were found in abundance on pebbles and shells and in the folds of the *Ulva*. Egg laying continued in the tide pools and aquaria through July and August, although in August there occurred an increasing number of apparently spent, large-sized individuals with reproductive system partially or completely absent. Thus, in 1937, the breeding season began in late June or early July and declined toward the end of August. Less extensive observations indicated that the same situation obtained during the season of 1936.

Discussion

The foregoing account of egg laying in *Polychoerus carmelensis* provides evidence in support of the hypothesis of von Graff and Bresslau that the eggs of some of the Acoela may be discharged through breaks in the body wall. *Convoluta roscoffensis* is the only other species of this group in which egg laying has been described as involving rupture of the parent worm. In that species, however, the body of the worm is frequently broken completely in two at the time of egg deposition. The regenerative powers of the Acoela have been demonstrated by the experiments of Stevens and Boring (1905) and of Child (1907) for the Pacific Coast *Polychoerus*, and by Keil (1929) for the Atlantic Coast *Polychoerus caudatus*. The healing of injured surfaces occurs remarkably rapidly in *Polychoerus carmelensis*. For example, when worms are cut from the posterior end almost the entire length of the body, the two almost separate halves are quickly brought into contact by their normal locomotor activities, and heal within about ten minutes. A small notch may remain at the posterior end, but frequently all trace of the cut is obliterated. For organisms which heal and regenerate so readily the emergence of eggs through rupture of the body wall, in the absence of oviducts, may be regarded as a natural and direct method.

SUMMARY

The egg-laying habits of *Polychoerus carmelensis* are described, with especial emphasis upon the path of emergence of the eggs.

The mature eggs pass from the two vitellaria ventrally and medially between the ventral integument and the bursa seminalis, and emerge to the exterior through a median rupture at the level of the bursa.

The jelly mass is secreted by the entire surface of the animal during repeated rotation, head over tail. The eggs emerge during rotation, probably aided by violent muscular contractions observable at this time.

The entire process of egg laying requires five or six minutes.

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THE EFFECT OF THE REMOVAL OF PERISARC ON REGENERATION IN TUBULARIA CROCEA

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Introduction

In 1903 Morgan observed that small pieces of Tubularia stems, which ordinarily regenerated hydranths at both ends, formed single hydranths at only one end when the other end of the stem was set upright in sand. Regardless of whether the distal or the proximal end was placed in the sand the exposed end was the one which formed the hydranth. Barth (1938*b*) extended these observations and showed that merely placing a glass capillary over the cut end of a stem would result in a complete inhibition of regeneration at the covered end. He also observed that when the coenosarc terminated a few millimeters below the level of the perisarc no regeneration occurred. It was suggested that oxygen lack was the probable cause for this inhibition, and that, in the last-mentioned case, the perisarc did not permit sufficient O_2 to reach the coenosarc for regeneration to occur. A logical experiment arising from these observations was one in which the perisarc was removed from a region along the length of the stem in order to expose the coenosarc to sea. water. Morgan (1903) found that when long slivers of the stem of Tubularia (both perisarc and coenosarc) were cut out, the portions of the stem adjacent to this cut regenerated in the same way that two separated portions of a stem would regenerate, i.e., a hydranth formed at either side of the cut, with the oral ends facing the cut region. The two hydranths so formed were connected at the oral ends during the initial stages of regeneration but separated after a while. Goetsch (1929) briefly reported that when all cut ends of a stem of *Pennaria* were sealed with wax, the removal of some of the perisarc was sufficient stimulus to cause regeneration at the exposed region. Peebles (1931) attempted to get lateral outgrowths from stems of Tubularia by "wounding the perisarc" but failed to do so. The present work was an attempt to work out in some detail the consequences of exposing the coenosarc in Tubularia. These experiments were performed in a preliminary way in the summer of 1937 (when it was found

that exposing the lateral coenosarc did result in hydranth formation, especially if the ends were ligatured) and were repeated and quantitative data gathered in the summer of 1938. I should like, at this point, to thank Professor Barth for his many suggestions which were so helpful throughout the course of these experiments.

Method

Essentially the same technique as the one described by Barth (1938*a*) was used for selecting the stems, keeping them, and for performing the necessary calculations. Straight, unbranched stems were selected from colonies of long (8–9 cm.) stems. These were selected for uniformity in thickness, etc. The hydranth plus 3–5 mm. of the stem was removed and the required length of stem was cut from the region immediately proximal to this. The ends were then ligatured as desired and the perisarc was removed. This removal was effected by means of a pair of iridectomy scissors used under a binocular microscope. Ligatured stems were selected at random for the various groups in an experiment. The desired measurements (the area of the exposed coenosarc and the diameter of the stem) were made with an ocular micrometer. The stems were kept on a gauze platform in a dish where aerated sea water was circulating constantly. To insure uniformity of conditions all of the stems involved in any one experiment were kept together in the same dish. The rate of regeneration was obtained by measuring the length of the regenerant at the time it was constricted from the rest of the stem, and employing the time required for this to occur as t_1 in the formula $R = \pi r_2 L/t_1$ (see Barth, 1938*a*) where r is the radius of the stem at the region involved, and L is the length of the primordium. In cases (Fig. 1—1 *C*) where the regenerant was not a complete cylinder the volume was obtained indirectly by employing a bit of rubber tubing as a model for the stem. A sector of the tubing equivalent in size and shape to the regenerant was cut out and weighed. It was found that this was from one-third to one-half the total weight of the tubing. The volume of the actual cylinder of stem was calculated using the longest length of the regenerant as L, and then the necessary one-third or one-half was subtracted from this figure. All of the data presented are averages for the various groups considered. Only stems showing regeneration within 72 hours after the beginning of the experiment were considered in the calculations.

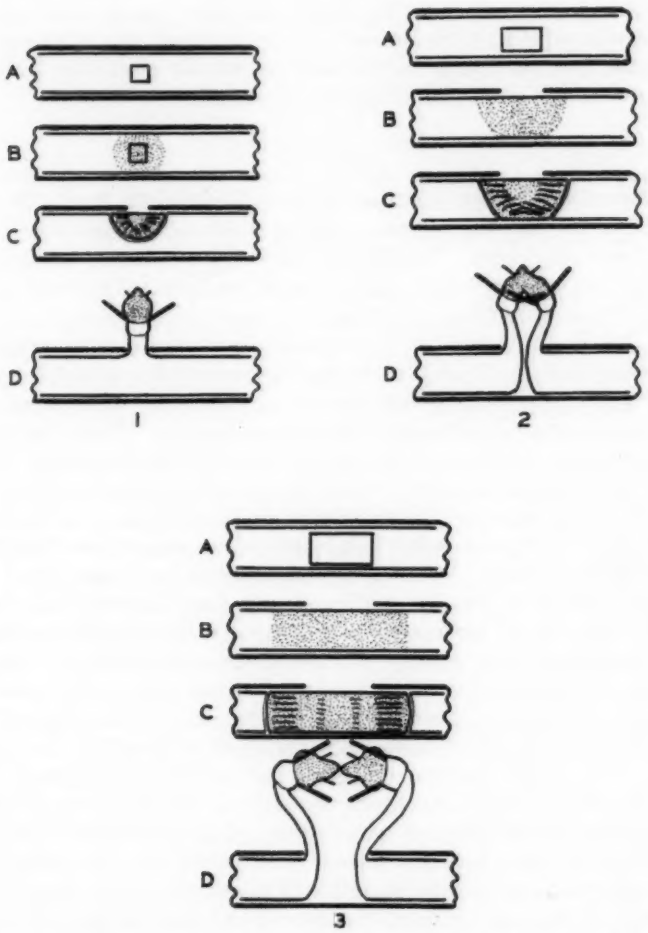


FIG. 1. Diagrams to show the effect of increasing the size of the exposed area upon the morphology of the regenerants. One, 2, and 3 are the three size groups. *A* indicates the amount of perisarc removed. *B* shows the beginning of pigment deposition. *C* shows the constriction of the regenerant from the rest of the stem and the appearance of the tentacle buds. *D* represents the condition of the regenerant after it has emerged.

Relation between the Area Exposed and the Rate of Regeneration

After preliminary experiments indicated that the exposure of the coenosarc was a sufficient stimulus for regeneration, an attempt was made to see whether there was any relation between the area exposed and the rate of regeneration. If, as was suspected, exposure to oxygen was the cause for regeneration a greater amount of exposed tissue should result in a larger regenerant. This was found to be the case. Table I presents data from two experiments which illustrate this. Ex-

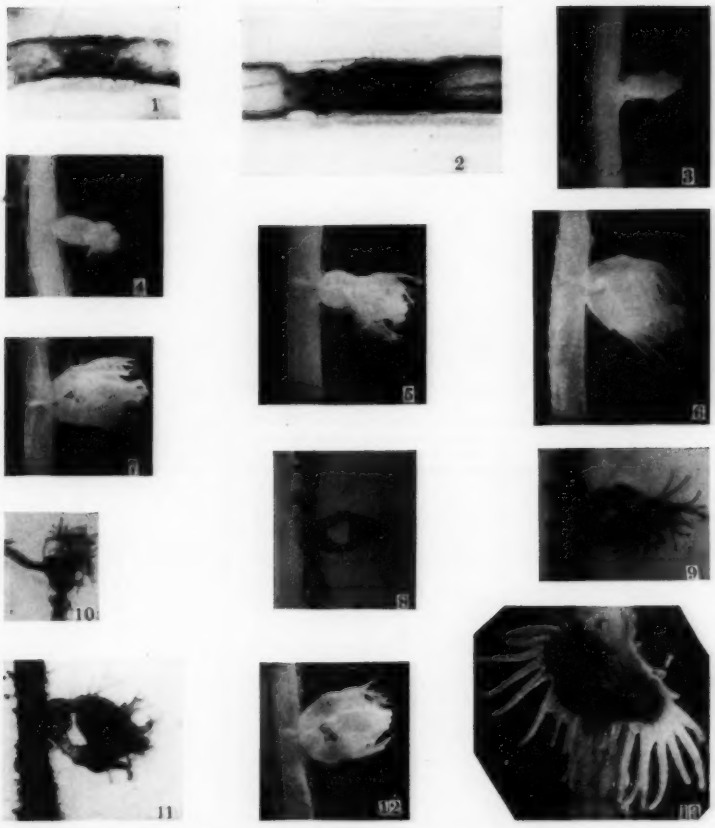
Table I

Fifteen-millimeter stems. *A*, *B* and *C* are the three size-groups for the areas exposed. *No.*, number of stems used; *Diam.*, diameter in micra; *Area* in square micra; T_1, time in hours; *No. Reg.*, number of regenerants; *Rate*, rate of regeneration.

	A	B	C
EXP. 1			
NO.	10	9	10
DIAM.	769	789	802
AREA	55073	153793	281559
T_1	43.7	48.1	47
NO. REG.	4	8	9
RATE	36	76	137
EXP. 2			
NO.	10	10	10
DIAM.	457	490	477
AREA	48152	132793	164962
T_1	38.5	38.6	32.4
NO. REG.	7	8	9
RATE	71	89	129

periment 1 was performed early in June when the stems were thick and experiment 2 was a repetition of the experiment performed in July when the young thin stems prevailed. The results are essentially the same as far as rate of regeneration is concerned. As the area of the exposed region was increased, the rate of regeneration went up. Since the average time in which this regeneration occurred was approximately the same for all of the groups in any one experiment, it can be safely assumed that it was the amount of tissue converted in a unit of time that was increased as more coenosarc was exposed. (See Barth, 1938*b*, where both t_1 and L vary with the oxygen tension.)

PLATE I



1. A central regenerant (as in Fig. 1—1*C*) at the constriction stage.
2. A central regenerant (as in Fig. 1—3*C*) before constriction. The tissue is slightly contracted.
3 and 4. Single hydranths (as in Fig. 1—1) after emergence.
5–12. Various degrees of fusion of the two emerged hydranths. (10 was cut from the stem at the point of emergence.)
13. Looking down on two complete hydranths which are connected at the hypostomes.

(Figs. 2 and 13 were photographed by Dr. A. N. Solberg.) (The author wishes to thank Mr. Jack Godrich for his assistance in preparing the graphic material for publication.)

Morphological and Polarity Variations

When thicker stems were used (Table I, Experiment 1 and preliminary) in the above experiment it was found that in addition to a variation in the rate of regeneration there was a variation in the morphology and in the polarity of the regenerants which depended roughly on the amount of tissue exposed. As depicted in Fig. 1 and in Plate I, there were three main categories of regenerants. When the exposed area was small (Fig. 1, case 1; Pl. I, 1, 3 and 4), only a limited amount of the coenosarc immediately surrounding it was involved in regeneration. In these cases the tissue involved in hydranth formation did not extend completely around the stem but formed a curved disc. When the proximal tentacle buds appeared they radiated out from the exposed central area. The distal tentacles frequently formed during the later stages of regeneration or during emergence. Such regenerants emerged as complete single hydranths whose polarity, i.e., oral-basal axis, was at right angles to the original polarity of the stem. In most of the cases where a greater amount of coenosarc was exposed (Fig. 1, case 2) the tissue involved in regeneration extended completely around the stem but was not symmetrical. A longer portion of the stem was involved in hydranth formation in regions immediately adjacent to the opening, while less and less of the stem was involved the farther it was removed from the opening. Such regenerants formed cylinders with oblique ends and had the appearance of trapezoids when they were viewed from the side (Fig. 1, case 2, *B* and *C*). These emerged as two more or less completely fused hydranths (Pl. I, 5 to 13). The extent of the fusion depended on the length of the region farthest from the opening. Where this was short the two hydranths were completely fused, as this increased in size the bases of the hydranths became separate but the oral portions were in common. All degrees of such fusion were encountered. The oral-basal axis in these cases was at various oblique angles to the original axis. In a few cases very large areas were exposed (the perisarc was removed from about one-half the surface of the stem for a length of 2–3 mm.) and a complete cylinder of coenosarc was involved in hydranth formation. The tentacle buds formed symmetrically around the stem (Fig. 1, case 3, *C*) and when the two hydranths emerged they were complete and mirror-images of each other. They remained fused at the hypostomal region and did not separate even when kept for 10 days. Here the original polarity was retained by the proximal regenerant and was completely reversed in the distal one. These results were similar to Morgan's cases (cited above) except that in his experiments the two hydranths separated. This difference is probably due to a mechanical factor, i.e., in Morgan's cases there was a thin bridge of tissue between

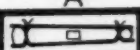
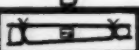
the two hydranths and this probably broke very easily when the regenerants contracted, while in the present cases there was a thicker connection which was not broken.

Injury to Tissue

In the light of Child's (1927*b*) experiments on *Corymorpha*, where laceration of the tissue (in this case not covered by a perisarc) was sufficient to cause regeneration, the work described above was open to the valid criticism that injury to the tissue and not exposure to the sea water was the stimulus for regeneration. The following experiment was performed to settle this question: Three groups of stems (Table II)

Table II

Fifteen-millimeter stems. *A* is the group in which the perisarc was removed without injury to the coenosarc. *B* is the group in which the perisarc was removed and the coenosarc was lacerated. *C* is the group where the coenosarc was lacerated and where the perisarc was replaced. Legend as in Table I.

	A	B	C
			
NO.	9	10	10
DIAM.	725	745	738
AREA	189488	177805	187638
$T_{\frac{1}{2}}$	45.5	47.4	–
NO. REG.	6	5	0
RATE	64.6	67.5	0

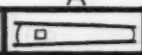
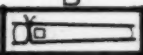
were selected and ligatured at both ends. The perisarc was removed very carefully in the first group and whenever there was any suspicion that coenosarc had been injured the stem was discarded. In the second group the coenosarc was deliberately lacerated after the perisarc had been removed. In the third group a three-sided flap was made in the perisarc and the underlying coenosarc was lacerated after the flap had been deflected. The flap of perisarc was then allowed to fall back into position—covering the lacerated tissue. Table II shows that the first two groups regenerated at approximately the same rates, while the third group did not regenerate at all. This shows conclusively that exposure to sea water is, in itself, a sufficient stimulus for the initiation of regeneration.

Dominance Relations

Since all of the experiments described above were performed on stems which were ligatured at both ends an attempt was made to see whether the open ends of a stem would affect the regeneration of an exposed region of that stem. Three experiments were performed in each of which four groups of 20 mm. stems were set up. In group A both ends were left undisturbed; in group B the distal 2–3 mm. were ligatured so that there was no continuity between that region and the rest of the coenosarc; in group *C* the proximal 2–3 mm. were ligatured; and in group D both ends were ligatured. Perisarc was then removed from the stem at various levels. In Table III the data for an experiment in

Table III

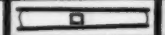
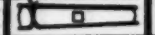
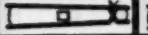
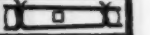
Twenty-millimeter stems. Showing the effects of the ends on a central regenerant. The openings indicated were 5 mm. from the distal ends. *A*, both ends open; *B*, the distal end ligatured; *C*, the proximal end tied off; *D*, both ends ligatured. Legend as in Table I.

	A	B	C	D
				
NO.	9	9	10	10
DIAM.	659	682	675	708
AREA	170053	145365	157562	192508
T_i	–	36.3	34.3	39
NO. REG.	0	3	3	4
RATE	0	57.7	34.9	115.5

which the perisarc was removed from a region 5 mm. from the distal end is presented. Table IV contains the data for an experiment in which the perisarc was removed from a region which was 10 mm. from the distal end; and Table V contains the data when the perisarc was removed from a region which was 15 mm. from the distal end. It is apparent, when these data are examined, that the ends, especially the distal end, exert a strong inhibiting influence (dominance) on the central regenerant when these ends are in continuity with the tissue of the stem. When this continuity is broken by a ligature the inhibition is no longer present and the rate of regeneration of the central region increases greatly. The proximal end by itself does not exert nearly as much of an inhibiting influence as does the distal. In Tables IV and V it can be seen that when only the distal end was tied off the rate of regeneration was much higher than when the proximal end was tied off.

TABLE IV

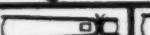
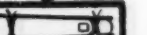
Twenty-millimeter stems. The opening is 10 mm. from the distal end. Rest of table as in Table III.

	A	B	C	D
				
NO.	10	9	10	9
DIAM.	632	586	603	597
AREA	184778	179731	165931	139379
T_1	44	56.6	52	50
NO. REG.	2	5	2	7
RATE	9.1	42.8	8.9	95.6

In fact the distal end exerted just about as much dominance by itself, in these cases, as did both ends combined. *C* in Tables IV and V is as low as *A*. In all three experiments there is a marked increase in the rate of regeneration when the inhibiting effect of both ends is removed. The one group which is in apparent discord with these data is group *C* in Table III. Here, despite the fact that the distal end is not liga-

TABLE V

Twenty-millimeter stems. The opening is 15 mm. from the distal end. Rest of table as in Table III.

	A	B	C	D
				
NO.	10	10	10	9
DIAM.	553	512	541	514
AREA	142567	117155	161352	111024
T_1	–	44.5	–	47.6
NO. REG.	0	6	0	9
RATE	0	39.3	0	68.9

tured, the rate of regeneration of the central region is fairly high. This is probably due to the fact that the perisarc was removed from a region which was fairly close to the distal end (appr. 5 mm.). The same factors responsible for the appearance of bipolar regenerants in small pieces of Tubularia are probably responsible for the high rate of regeneration of the central regenerants in this group. This has been explained (Morgan, 1901) as being due to the fact that these tissues are very

close together and are probably metabolizing at about the same level and thus do not inhibit each other. Whether or not this is the case, the evidence still indicates definitely that the ligaturing of the ends will result in a great increase in the rate of regeneration of a central exposed region and that the ends do exert a dominance over a central region when they retain continuity with such a region.

Finally, an experiment was performed to determine whether dominance was exerted by a more distally placed exposed region over a more proximally placed exposed region. In Table VI we can see that

TABLE VI

Fifteen-millimeter stems. Both ends ligatured. *A*, the exposed region at the distal part of the stem; *B*, the exposed region at the proximal end of the stem; *C*, an exposed region at both ends. Legend as in Table I.

	A	B	C	
			D	P
NO.	10	10	10	
DIAM.	503	514	521	538
AREA	149021	140021	163738	173309
T_1	42.4	48.6	44.7	–
NO. REG.	5	6	7	0
RATE	41.3	45.9	60.2	0

this is definitely the case. In this experiment 15 mm. stems were used and both ends were ligatured close to the cut surfaces. The two exposed regions were separated by about 10 mm. The distal exposed area, though slightly smaller, completely inhibited the proximal one. Groups *A* and *B* in this table serve as the controls.

DISCUSSION

For the first few hours after its exposure the coenosarc of Tubularia is in a condition approximating the early indeterminate stages of an embryo (e.g., the ectoderm of an amphibian embryo). There is a tendency to continue its usual vegetative activity and produce more perisarc to replace that which has been removed. Sooner or later, however, what seems to be a competition between the tendency to retain the vegetative state and the tendency to form hydranth material begins to manifest itself. A visible evidence of this is the appearance of the pink pigment which typifies the first stage in regeneration. Now, de-

pending on the existing conditions (size of the opening, dominance of another region, or inherent conditions within the stem), the coenosarc either becomes definitely determined to form hydranth tissue or retains its original condition. In the former case a hydranth forms and eventually breaks through the thin perisarc which had been secreted in the earlier stages. In the latter the pigment disappears after a while and the coenosarc continues to produce more perisarc until the site of the removal cannot be distinguished from its surroundings. Occasionally, when the opening is very small or when another region is dominating it, the exposed coenosarc does not even begin to regenerate (i.e., no pink pigment is deposited) but continues to form more perisarc as though there had been no interruption. The analogy to amphibian material is made more striking when it is shown that there is a period after which the determination of the hydranth-forming material becomes irreversible. This has been shown by Peebles (1931) and by another method by the author in some unpublished preliminary work. The analogy breaks down, however, when the stimulus for the determination is considered. Although the primary stimulus for form and organ formation in amphibian embryos may come from an outside source, the work of the past few decades has indicated that the immediate cause of determination of a given structure comes from adjacent tissues. In Tubularia, as well as in many other hydroids (Child, 1927*a*) the stimulus for hydranth formation comes from outside of the organism. (The various parts of the hydranth may be determined as in the Amphibia). Many external agents have been cited (Child, 1929) as the stimulators for regeneration.

In referring to the experiments in which stems of Tubularia were either placed end down in sand or in which the end was capped with a glass capillary, both Morgan and Barth suggested that an oxygen lack might be responsible for the ensuing inhibition. It was implied that the presence of sufficient O_2 would allow regeneration to proceed. Accumulated wastes rather than O_2 lack was not excluded as a possible cause of the inhibition. The present experiments do exclude this since the sea water in which the stems were kept was well aerated and was kept in constant circulation (the glass-capping experiments were repeated under these conditions and the same results were obtained). It seems likely, therefore, that the inhibition of regeneration is due to oxygen lack; and that when an adequate supply of O_2 reaches the tissue it serves as a stimulus for regeneration. This idea is rendered more plausible when the effects of varying the oxygen tension on the rate of regeneration are considered. Barth (1938*b*) found that both the length of the regenerant increased and that the time in which constriction of the re-

generant occurred decreased as the oxygen tension was increased. This indicates that as the only variable in these experiments, i.e., oxygen, was increased a greater amount of tissue was converted into hydranth tissue in each unit of time. This bears a definite similarity to the experiments cited above in which the size of the regenerant increased when more coenosarc was exposed to the influence of the sea water and suggests a similar causal relationship. The similarity between the action of oxygen and the exposure of the coenosarc is made more apparent by some unpublished work of Dr. J. A. Miller. (Dr. Miller was kind enough to grant the author permission to mention some of his material in relation with the present work.) This work of Miller's concerns the effects obtained with varying the oxygen tension of the sea water in which small bits of Tubularia stem were kept. These small stems ordinarily yield bipolar regenerants. As the tension of the oxygen was increased, the number of incomplete bipolars was increased. This indicates that the various regions of the regenerant increased in size and that the central portion of the small stem was used for more distal structures instead of for the more basal regions which they would have formed in a lower concentration of oxygen. Since this increase in primordia size occurred equally at both ends, there was not enough material to form complete hydranths and two symmetrical incomplete forms resulted—more or less of the basal regions were missing. These results are, to some extent, the converse of the results obtained when the perisarc was removed from the stem. When the area exposed was small the regenerant was small and a single complete hydranth formed,—just as the bipolars were usually small complete hydranths when the oxygen tension was low. When the openings were sufficiently large more tissue was involved and the regenerants were enlarged and were more complete. When Miller increased the oxygen tension the bipolars became more incomplete for the same reason, i.e., the regenerants became enlarged but at the expense of their basal regions. The striking similarity between the effects of exposing the coenosarc and increasing the oxygen tension leads one to conclude that the former is also probably the effect of the oxygen in the sea water. The experiment where the perisarc was replaced helps to substantiate this.

If the premise that an increased oxygen supply stimulates the coenosarc to form hydranth tissue is granted, then the results obtained in these experiments can be explained very readily. The coenosarc must be metabolizing at a certain level before hydranth tissue will form. When a small opening is made in the perisarc only the tissues immediately around the opening get enough O_2 to raise them to this level. Since the basal portions of the hydranth (including the proximal ten-

tacles) form at the lower level of activity, and since this lower level is symmetrical around the opening, a small but complete hydranth forms. But, since the region exposed to the oxygen is at the side, and the high activity level is around this, the new hydranth's polarity is at right angles to the polarity of the stem. The other form and polarity variations may be explained in the same way. As more oxygen is admitted into the stem, more of the coenosarc is activated to form hydranth tissue and, depending on the resulting gradient of oxygen, the two hydranths produced are more or less fused. If a small region of the stem opposite the opening is activated the basal regions as well as the distal will be fused. If a greater region of this part of the stem is activated the basal portions of the hydranth will be separate. And as this region increases in length more and more of the hydranths become separated until two complete forms are produced.

The work on dominance does not throw any new light on the factors involved in this phenomenon, but simply illustrates the inhibition exerted by the ends upon a central region.

Summary

1. Experiments in which lateral regions of the coenosarc of Tubularia were exposed indicate that the rate of regeneration in such regions is proportional to the area exposed to the influence of freely circulating sea water.

2. The morphology and polarity of central regenerants in thicker stems varies with the area exposed. Forms ranging from single complete hydranths, whose polarity was at right angles to the original polarity, to two hydranths, fused to a greater or lesser extent, were obtained. The polarity of the double hydranths varied with the degree of fusion. All angles to the original polarity were obtained.

3. Injury to the exposed coenosarc is not a factor in the present cases.

4. The ends of a stem exert a definite dominance over the exposed central regions. This dominance may be removed by ligaturing the ends. When this is done the rate of regeneration of the central region increases greatly.

5. A more distally placed central exposed region inhibits another exposed region placed more proximally on the same stem.

6. It is suggested that the probable stimulator of regeneration in Tubularia is the oxygen in the sea water. The results on rate of regeneration and on morphological and polarity variations may be explained by the amount of oxygen available to the coenosarc when areas of different size are exposed.

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THE RÔLE OF TEMPERATURE IN HYDRANTH FORMATION IN TUBULARIA

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Tubularia crocea (Agassiz) is a hydroid found on wharfs and piles in the Woods Hole region. It is abundant until the last part of July when the hydranths are dropped and the coenosarc retreats into the perisarc. The colonies remain in this condition until October when growth begins anew. The experiments reported in this paper show that temperature is an important factor in regulating this periodicity.

Autotomy and resorption of hydranths under a variety of conditions have been studied by a number of investigators, for example, Bäschlin (1932), Cerfontaine (1903), Gast and Godlewski (1903), Godlewski (1904), Huxley and deBeer (1923), Loeb (1900), Morse (1909), Riddle (1911), and Thatcher (1903). Morse (1909) made a special study of the cause of autotomy of the hydranth in *Tubularia* and concluded that ". . . temperature seems to be the only consistent factor involved in the decapitation. When the temperature is kept at about 10° C. or 15° C., the hydranths are retained, regardless of any other factors with the one exception of lack of oxygen, which we believe to be inoperative except under wholly artificial conditions." Sumner, Osburn and Cole (1913) recorded the disappearance of *Tubularia crocea* during the summer from the harbor at Woods Hole but noticed that in cooler waters the colonies may remain in an active condition throughout this period. Elmhirst (1922) kept colonies of *Tubularia* in his aquarium for three years and noticed that hydranths appeared in midwinter and were lost in midsummer. Light and oxygen were thought to be the principal factors involved.

If high environmental temperatures cause *Tubularia* to exist in a dormant condition, then keeping them in colder water should result in renewed growth. It was also thought that oxygen might be a factor since its importance in regeneration has been demonstrated by Barth (1937, 1938) and Miller (1937). The following experiments were performed to test the action of temperature and oxygen on dormant colonies of *Tubularia*.

Experiments

On August 6, 1938 an experiment was begun to test the effect of oxygen and temperature on dormant colonies. Two 500 cc. flasks were placed in an 18.4° constant temperature bath. Into one of these, *A*, air was bubbled continuously at the rate of 1 liter per minute. The other, *B*, received oxygen at the rate of 1 liter per 11–14 minutes. These gases kept the water saturated and in rapid motion. Two similar flasks were placed in running salt water which varied from 23.2° to 24° for the duration of the experiment. (Sea water in laboratory tanks is from 0° to 1° warmer than where *Tubularia* grows.) One of these, *C*, received air at the same rate as *A*. The other, *D*, received oxygen at the same rate as *B*. Dormant colonies were placed in these containers. After 72 hours there were many hydranths in *B*, less in *A*, and none in *C* and *D*. At 96 hours nearly every free end of the *B* colony had a fully formed hydranth. In *A* they were not so numerous. *D* showed but two small hydranths (occasionally a hydranth is noticed on dormant colonies as they are collected), and *C* showed none.

On August 18, 1938, colonies were collected and placed in flasks as in the previous experiment. The temperature of laboratory ocean water had fallen to 22° C. The experiment was terminated August 24,

Table I

See text for explanation.

Flask	Temp.	O_2	Total hydranths	Hydranths/gm. colony
	°C.	cc./l.		
A	18.4	5.5	243	128
B	18.4	23.5	116	83
C	22.	5.0	0	0
D	22.	21.3	31	38

the colonies removed from the flasks and the number of hydranths carefully counted under the binocular microscope. The oxygen tension in each flask was determined by the Winkler Method. Excess water was removed from the colonies and their weight determined. The results are shown in Table I.

Discussion

Morse (1909) found that *Tubularia* disappeared when the temperature of ocean water rose to 20° C. We observed that in the summer of 1938 it disappeared when the water temperature was 21°. From Morse's experiments and those here reported it seems highly probable

that autotomy of hydranths and the existence of the colonies in a dormant condition is due to high temperature. Conversely, hydranths will be produced in abundance at a few degrees below 20°. North of Cape Cod where the water does not rise above the critical temperature for *Tubularia,* colonies flourish throughout the summer months (Morse, 1909).

This existence of coelenterates in a state of dormancy in regions where conditions during part of the year do not permit active growth has been pointed out by Broch (1925), who called attention to its similarity to spores in plants.

It is doubtful if oxygen in the concentrations normally present in the sea plays a significant rôle in the disappearance or reappearance of active colonies of *Tubularia.* However, in flask D (Table I), kept at 22°, a number of hydranths were formed. The oxygen tension was far above that ever present in the ocean. On bright days the oxygen tension of water bathing the dormant colonies has measured 6.1 cc. per liter, higher than in either flasks *A* or *C* (Table I) yet there is no renewed growth as long as the temperature is over 20° C. In the experiment conducted at 24° saturation with oxygen failed to produce a significant number of hydranths. It appears that at temperatures just above the critical point a high oxygen tension may stimulate some hydranth formation but this effect is not noticed at still higher temperatures.

The author wishes to thank the Department of Zoölogy of Columbia University for providing facilities for investigating this problem at Woods Hole and Professor L. G. Barth for his helpful suggestions.

Summary

When ocean water rises above 20–21° C. in the Woods Hole region the hydranths of *Tubularia crocea* are lost and the coenosarc retreats into the perisarc. The colonies remain in this dormant condition until the temperature drops in autumn. Growth is then resumed.

The dormant colonies can be stimulated to produce hydranths in three days by keeping them at 18.4° C.

It is thought that temperature is the principal agent in the disappearance and reappearance of active colonies.

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STANDARDIZATION OF THE PRECIPITIN TECHNIQUE AND ITS APPLICATION TO STUDIES OF RELATIONSHIPS IN MAMMALS, BIRDS AND REPTILES

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Serologic techniques are now used in studies of relationships among bacteria, plants (see review by Chester, 1937) and animals. The types of tests commonly employed are the complement fixation, the agglutination and the precipitation reactions. The particular type of study governs the test employed and there are advantages to each one.

Although it is claimed that the precipitin reaction is not as sensitive as other serologic methods, its simplicity and accuracy make it advantageous in studies of animal relationships where large numbers of tests are necessary. The inconsistency of results, as shown in serologic literature, is possibly not due to the unreliability of the precipitin reaction but rather to the differing techniques of the workers who make the tests. While the techniques that were first employed in the precipitin reaction were important and gave interesting results, they have been much improved in recent years and need still further standardization. There should be a uniform method of measuring the end-point (titer), the chemical and physical factors should be constant and the methods of injection should be standardized.

The precipitin reaction is the one that has been most commonly used in the study of animal kinship, though agglutinins, responsible for blood groups, have been employed in the case of apes and man. Comparatively little research has been done on the serological relationships of reptiles or of birds. Nuttall (1904) stated that there was "a similarity of the blood constitution of all birds." He found differences in degree of reactions but these were so slight that a study like that made with mammalian bloods was not possible. Sasaki (1928) claims that he was able to distinguish the Japanese duck (*Anas domestica erecta*) from the Chinese goose (*Anser cygnoides*) with untreated sera and could distinguish the Japanese duck from the Muscovy duck (*Cairina moschata*) by specialized sera. These results would be more reliable were the possible error of the tests determined. Graham-Smith (1904—see Nuttall, Section VIII) found a closeness of relationship between the Crocodilia and

Chelonia and between the Ophidia and Lacertidia. The reactions indicated that the former groups stood more closely related to the Aves.

Ehrhardt (1929) reviewed the whole field very completely. Some important research has been done recently showing the significance of the tests in determining uncertain relationships. Boyden and Noble (1933) applied the reaction to a study of relationship among several amphibians and clarified the status of these forms. Eisenbrandt's (1938) extension of these methods to chiefly parasitic helminthes is of value in showing that lower animals with only relatively small amounts of circulating proteins may also be successfully studied.

Zuckerman and Sudermann (1935) reported on "Serum relationships within the family Cercopithecidae." As part of their conclusions they state, ". . . their findings support the view . . . that the serum-precipitin test is of limited value in tracing phylogenetic relationships." Their conclusions from their data are not justifiable, however, since these data were obtained by a technique which is questionable.

Magnus (1908) noted that antisera specificity was lessened by continued injections of an antigen. Wolfe (1935, 1936) and Wolfe and Baier (1938) emphasized the importance of the effects of injection methods on the specificity and aspecificity of the serum precipitin tests. It was shown that the type of response is greatly influenced by the method of injection, the specificity being enhanced by limiting the amount of protein injected. With this technique they found greater distinctions among more closely related forms and less "extra-group" reactions. These results suggested that even greater specificity might be possible if the amount of protein injected was reduced to a minimum. It is known that exceedingly small quantities of protein may cause the formation of precipitins in rabbits. Hektoen and Cole (1932) were able to produce antisera of fairly high titers in rabbits by injecting as low as .00029 gram of an ovalbumin solution.

The purpose of this research is: (1) to determine whether precipitin specificity can be increased by limiting the quantity of protein injected into rabbits to a minimum necessary for antibody production; (2) to discover whether a serological relationship study on birds and reptiles is practicable; (3) to attempt to further standardize precipitin technique.

Methods and Materials

Healthy adult rabbits of mixed breeds and weighing approximately three kilograms were injected intravenously with serum proteins of ten species of mammals, five species of birds and one species of reptiles. Listed in Table I are the scientific and common names of the animals

TABLE I

Antigens used in experiments

Scientific Name	Common Name	Grams protein per 100 cc. serum
Homo sapiens *	Man	6.843
Pans satyrus	Chimpanzee	6.677
Macacus rhesus	Rhesus macaque	5.425
Macacus sinicus	Bonnet macaque	6.926
Papio hamadryas	Hamadryas baboon	5.530
Lasiopyga mona	Mona guenon	8.616
Cebus capunicus	Capuchin monkey	7.198
Bos taurus *	Ox	6.825
Bison bison *	American buffalo	7.982
Capra hircus *	Domestic goat	6.593
Ovies aries	Domestic sheep	6.051
Odocoileus virginianus	White-tailed deer	5.843
Tamiasciurus hudsonicus *	Red squirrel	6.409
Sciurus carolinensis *	Gray squirrel	8.750
Sciurus niger	Fox squirrel	6.560
Citellus tridecemlineatus	13-lined ground squirrel	5.096
Tamias striatus	Eastern chipmunk	5.139
Marmota monax	Ground hog	6.260
Rattus norvegicus	White rat	7.052
Felis domesticus	Cat	6.825
Canis familiaris	Dog	6.364
Gallus domesticus *	Chicken	4.676
Columba livia *	Pigeon	3.133
Strix varia	Barred owl	4.070
Larus argentatus	Herring gull	3.070
Ardea herodias	Great blue heron	3.550
Mergus merganser	American merganser	3.800
Anser anser	Domestic goose	4.387
Anas boschas	Domestic duck	4.676
Alligator mississippensis	Alligator	5.158
Phrynosoma cornutum	Texas horned toad	4.389
Chelydra serpentina *	Snapping turtle	3.654
Chrysemys picta	Painted turtle	3.446
Emys blandingii	Blanding turtle	2.299
Clemys insculpta	Wood turtle	3.550
Thamnophis sertalis	Common garter snake	5.405

* Used for production of antisera and test antigens, others only for test antigens.

from which the blood proteins were secured. The amount of protein per 100 cc. was determined on the whole serum by the Folin-Wright (1919) modified macro-Kjeldahl method. Corrections were not made for non-protein nitrogen. Table II records antisera production data for some of these species. The quantity of protein injected and titers are based on protein calculated from total nitrogen.

The amount of protein injected per animal in the first series has been as low as .000786 gram. This is an exceedingly small amount and not always do such quantities injected into rabbits yield potent antisera. When a second series of injections was given the dosage was usually about one-half the initial series dosage. At present a standardized injection technique is being followed. Rabbits are injected with .001 gram

TABLE II

Antisera production data

Rabbit No.	Serum protein injected	Injected dil. of solution	Total protein injected	Bled—days after last injection	Titer (dil.)
		cc.	*grams*		
205	Red squirrel	6.0 (2%)	.00768	12	512,000
215		1.2 (2%)	.001536	12	512,000
136		3.2 (1 : 1)	.102544	14	51,200*
217	Gray squirrel	1.0 (1 : 1000)	.001	10	0
		1.0 (1 : 1000)	.001	12	16,000
		0.75 (1 : 1000)	.0075	8	512,000
223		2.0 (1 : 1000)	.002	10	128,000
125		2.25 (undil.)	.19687	10	51,200*
A11	Ox	1.5 (1 : 500)	.003	2	0
				4	0
				6	16,000
				9	1,024,000
A 9	Buffalo	1 mg./kil.	.0034	10	256,000
A10	Goat	1 mg./kil.	.0034	10	128,000
185	Human	5.75 (2%)	.00786	12	51,200*
77		3.0 (undil.)	.20531	12	102,400*
209		0.6 (2%)	.00082	13	128,000
221		2.0 (1 : 1000)	.002	10	512,000
225	Chicken	2.0 (1 : 1000)	.002	12	0
		0.6 (1 : 500)	.0012	7	512,000
228		0.75 (1 : 1000)	.0015	8	1,024,000
229	Pigeon	1.0 (1 : 500)	.002	10	512,000
222	Snapping turtle	1.0 (1 : 500)	.002	10	0
		0.6 (1 : 500)	.0012	7	256,000

* This notation indicates a serial dilution from a 2 per cent standard—all the rest are from standard solutions containing .001 or .002 gram per cc.

of protein per kilogram body weight distributed over three injections. The injections are made on alternate days. When additional series are necessary the amount administered is one-half that of the initial series given in two injections on alternate days.

Ring tests were used exclusively in determination of the titer. The test consisted of having a duplicate series of serological tubes containing

0.5 cc. of antigen in serial dilution with 0.1 cc. of antiserum layered beneath the antigen. The titer was determined after incubation in a water bath at a temperature of 37.5° ± 1° C. The possible error in reading the reactions was ± 1 tube. Several experiments consisted of readings at intervals during the sixty minutes in order to study the progression of the titers. Unless definitely specified, the antisera were used undiluted or 1.0 cc. was diluted with 0.25 cc. of a buffered physiological saline solution. This small dilution did not have any effect on the strength of titers in the experiments.

In the earlier experiments the serial dilutions were made from standard test antigens which were 2 per cent solutions. The latter were made by diluting 1.0 cc. of the undiluted blood with 49.0 cc. of buffered saline giving a 1 : 50 dilution. Since the protein content of the antigens varied from 4 per cent to 9 per cent the titers need adjustment. In the later experiments all serial dilutions are made from standard solutions that contain either .002 or .001 gram protein per cc. thus making all titers comparable.

TABLE III

Anti-human sera

Antigen serum proteins of	77	185*	209	221
Man	102,400	51,200	128,000	512,000
Chimpanzee	25,600	12,800	128,000	256,000
Rhesus macaque	12,800	1,200	0	64,000
Bonnet macaque	6,400	1,600	0	64,000
Baboon	25,600	400	0	256,000
Mona guenon	12,800	400		128,000
Capuchin monkey				16,000
Rodentia		0		0
Artiodactyla	0	0	0	4,000
Carnivora	0	0		1,000

* Titers from a 2 per cent solution of undiluted antigen.

EXPERIMENTAL DATA

Effects of Amount of Antigen Injected on the Specificity of the Antisera Produced

One of the main purposes of this paper is to show the variance in the degree of the homologous and heterologous precipitin reactions when one factor, the injection procedure, is varied. Antisera 77, 185, 209, 221 are anti-human sera whose reactions are recorded in Table III. Serum No. 77 was produced by injection of .21594 gram protein while the rabbit producing No. 209 received only .00082 gram of protein. (Reference

should be made to Table II for exact detail on quantity of material injected in the experiments to be described.) Of the four antisera 209 is by far the most specific, giving reactions only with chimpanzee and human blood antigens. No. 185 is more specific than 221 even though the latter received one-third the amount of protein given the former.

Injections of the more minute quantities (similar to 185 and 209) usually gave reactions illustrated by these two and more rarely reactions like 221. On the other hand, when 3 cc. of undiluted serum was injected, the most usual response was like that given by No. 77.

Sera 77 and 221 yielded primate group reactions but No. 185 resulted in a sub-grouping showing that the blood proteins of man and chimpanzee are more nearly related to each other than to the blood of the monkeys. Serum 209 was an extremely specific one, giving reactions with only chimpanzee and the homologous antigens. Such antisera would be of great value to the medico-legal workers, as well as to the student dealing with man's closest relatives.

The reactions of three anti-red squirrel and three anti-gray squirrel sera are shown in Table IV. Serum 136 gave a Sciuridae group reac-

TABLE IV

Anti-squirrel sera

Antigen serum proteins of	Anti-red squirrel			Anti-gray squirrel		
	136*	205	215	125*	217	223
Red squirrel	51,200	512,000	512,000	2,400	64,000	0
Gray squirrel	25,600	0	128,000	51,200	512,000	128,000
Fox squirrel	51,200	0	128,000	51,200	256,000	2,000
13-lined gray squirrel	12,800	0	0	2,400	4,000	0
Chipmunk	51,200	0	0	6,400	8,000	0
Ground hog	51,200	0	0	3,200		0
Rat	0	0		0	0	0
Artiodactyla	400		0	200	4,000	0
Primata	400			400	4,000	0

* Titers from a 2 per cent solution of undiluted antigen.

tion with no sub-grouping evident, but No. 125, an anti-gray squirrel serum, reacted much more weakly with the blood of red squirrel and other sciurids but could not be differentiated from fox squirrel blood. Both these sera were produced by injection of a much larger quantity of protein than were the other four listed in the table. The latter all show that a definite difference exists between red squirrel blood and that of the fox or gray squirrel bloods. Sera 215 and 217 subdivide the Sciuridae reactions into three sub-groups. Serum 205 is very specific and gave no cross reactions at all and No. 223 allowed for distinction between the

TABLE V

Anti-aves sera *

Antigen serum protein of	Anti-chicken		Anti-pigeon
	225	228	229
Chicken	512,000	1,024,000	0
Pigeon	4,000	256,000	512,000
Goose	16,000	256,000	0
Duck	8,000	256,000	0
Merganser	0		
Great blue heron	8,000	0	0
Barred owl	8,000		
Herring gull	0		0
Snapping turtle	2,000		0
Painted turtle	0	0	0
Alligator	0	0	
Common garter snake	0	0	0
Cryst. egg albumen of chicken	0	0	0

* Fowl and reptile data are from a thesis by Miss Bernice Cohen done under the supervision of the author and used with her permission.

two closely related forms of gray and fox squirrels. This latter result is very rarely secured by the precipitin test with native antisera.

The variation in results that were secured with different dosages do not subtract from the value of the precipitin test but, on the other hand, give it greater possibilities. The differences in reactions seem to imply that a distinction can be made among genera within a family and possibly

TABLE VI

Anti-snapping turtle

Antigen-serum Protein of	*No. 222*
Snapping turtle	256,000
Painted turtle	64,000
Blanding turtle	16,000
Wood turtle	16,000
Alligator	0
Horned toad	0
Common garter snake	0
Aves (7 species)	0
Cryst. egg albumen of chicken	0

species within a genus as well as among larger groups. Similar results have been reported previously but there now seems to be a method whereby these results can be secured consistently. It would be advisable to have many antisera for each group studied in order to insure significant results.

It might be stated that it is believed that the variability of results is not attributable to the innate characteristics of the test but is probably

TABLE VII

Effects of Length of Incubation Period and of Dilution of Antisera

	Serum Undiluted					Dilution	
						1 : 3	1 : 5
Incubation Period—Minutes	1	5	10	30	60	60	60
		Anti-buffalo—A9					
Buffalo	0	64,000	128,000	128,000	256,000	512,000	128,000
Ox	0	64,000	128,000	128,000	256,000	512,000	128,000
Sheep	0	0	0	4,000*	64,000	4,000	1,000*
Goat	0	0	0	4,000	128,000	4,000	2,000*
Deer	0	0	0	32,000	128,000	4,000	
		Anti-goat—A10					
Goat	0	0	0	64,000	128,000	0	
Sheep	0	0	0	64,000	64,000	1,000	
Buffalo	0	0	0	0	0		
Ox	0	0	0	0	0		
Deer					0		
		Anti-beef—A11					
Beef	64,000	128,000	512,000	1,024,000	1,024,000	512,000	512,000
Buffalo	64,000	128,000	512,000	1,024,000	1,024,000	256,000	256,000
Sheep	0	0	0	16,000	128,000	0	
Goat					128,000	0	
Deer	0	0	0	64,000	256,000	0	

* Readings not clear.

due to the antibody producer, in this case, the rabbit. It would be an ideal condition were one able to secure a group of rabbits with similar potentialities for antibody production.

Tests with Avian and Reptilian Blood Sera

There is a meagerness of serological data with respect to reptilian and avain bloods probably due to the conception that the animals within each of these groups are very closely related to each other and are not suitable for study by means of the precipitin reaction. The data listed in Tables V and VI contradict this contention. There are indications that the reactions of anti-avian and anti-reptilian sera produced by using very small amounts of proteins may be useful in confirming present morphological data and thus aiding in the clarification of disputed relationships.

Effects of Incubation Period and Dilution of Antisera on Titers

Previous workers have considered such factors as the length of incubation period and the effect of dilution of the antisera on the homologous and heterologous reactions. The usual method is to make readings after 1 hour incubation at a temperature of 37° ± 1° C. when the ring test is used and the antisera are used undiluted or diluted to various degrees.

Table VII shows the reactions of three anti-Artiodactyla sera which illustrate both time and dilution effects. These antisera readings at 5, 10, and 30 minutes usually showed much greater specificity than did the readings at 60 minutes. Serum A 9 lost its distinguishing characteristic at 60 minutes so that the reactions of buffalo and ox could not be distinguished from deer and goat. It should be noted that antisera A 10 and A 11 show that beef or buffalo can be markedly differentiated from either goat or sheep bloods even at 1-hour readings. Heretofore these great differences have not been shown (with rare exceptions) when undiluted and untreated antisera against the serum proteins were used. By using very small quantities of blood for injections, results similar to, or showing greater differentiation, are consistently secured.

The dilution of antisera with physiological saline is first noted to have an effect on the antigens more distantly related (according to taxonomical schemes). These antisera became much more specific by diluting them as low as 1:3 (2 parts saline and 1 part antiserum). With beef antiserum A11 the reactions of sheep, goat and deer antigens disappeared at antiserum dilutions of 1:3 while the buffalo antigen titer remained similar to the homologous reaction. Such low dilutions show these effects only when the antisera have been produced by the methods described; that is, injections of minute quantities of proteins.

Discussion

Data are presented that show how a greater uniformity of results may be obtained with the serum precipitin reaction. These data further supplement previous work of the author in regard to the specificity and aspecificity of the test. The consistency of the specificity of the reaction with the whole serum proteins of mammalian, avian and reptilian bloods is greater than heretofore reported with untreated rabbit antisera. A distinction of the bloods of such closely related forms as ox and sheep, red squirrel and gray squirrel, several species of turtles and of a number of birds was possible.

The cause of this greater specificity is believed to be the small quantity of protein injected. A standardized quantity is now used; the amount injected in the first series is .001 gram of protein (based on total nitrogen) per kilogram body weight and distributed in three injections. If the titer of the antiserum resulting is insufficient the animal is reinjected with .0005 gram per kilogram body weight given in two injections. This amount injected is thought to be approximately the smallest amount of the antigen used that will regularly cause antibody formation.

The results are not always similar and therefore it seems necessary to produce a number of antisera against a particular antigen in studying relationships and choose those which have the desired specificity. With the present procedures employed the antisera against a particular kind of antigen fall into groups. The reactions of the different groups differ quantitatively but not necessarily qualitatively. Not only are relationships shown but the degree of such relationships can be studied with greater accuracy than before. Morphology does not allow for such a type of approach to the classification of animals. These differences can best be understood by referring to the tables on rodent or primate reactions.

The high specificity secured with a few of the antisera even when used untreated should interest the worker concerned with the medicolegal aspects. Antisera that give reactions only with very closely related bloods should be of much value and no doubt the evidence so secured would be readily accepted by our courts. Such antisera are comparatively easy to produce if the amount of protein injected is reduced to the approximate minimum.

The titer of the reaction at 1 hour incubation did not show the marked differences that were shown after incubating for shorter periods. This factor, the length of incubation period, has not been used with any uniformity for distinguishing closely related proteins. When antisera are produced by the injection of very minute quantities of protein the homologous blood and very closely related ones give ring tests with high titers after 1, 5, 10 or 20 minutes incubation and after that period the blood of more distantly related forms becomes positive. These reactions, when properly performed, are usually very distinct and consistent.

Attempts were made to use readings after incubation of more than 1 hour. These readings are not so clear-cut and not consistent and so are considered unreliable.

The second *in vitro* factor which enhanced the specificity of the reaction, that is, dilution of the antisera, showed a direct correlation with

the results secured by studying the period of incubation. Boor and Hektoen (1930) and Hektoen and Boor (1931), using antihemoglobin sera, found that dilutions with 3 parts of saline or normal rabbit serum eliminated extra specific reactions. Satoh (1933) demonstrated that dilution of 1 : 10 or less affects the titer of an antiserum when it is produced by few injections with a small quantity of material, and dilution of 1 : 25 or more affects the reactions when the antisera result from injection of larger quantities given in a greater number of injections. In Satoh's report the dilution seemed to have a similar effect upon the heterologous and homologous reactions. Wolfe (1933) reported that dilutions of 1 : 2 make the readings of the " ring " clearer but that dilutions of 1 : 10 do not always eliminate extra-group reactions. These antisera were produced by injection of a relatively large quantity of antigen.

The antisera that were used in the present work were easily affected by small dilutions with buffered saline. The lowest dilution to have an effect was a 1 to 1 (1 part saline) dilution but with some antisera it was necessary to dilute with 2 or 3 parts of saline in order to show decrease in reaction. The dilution always lowered or eliminated the heterologous titer first and one could thus verify the dissimilarities between such closely related bloods as ox and sheep, or man and monkey. But even a higher dilution could not distinguish consistently between ox and buffalo or man and chimpanzee for the dilutions had similar effects upon both titers.

The data presented in this paper are fairly consistent. The methods reported are easily followed and it seems they insure a uniformity of results. The different approaches used resulted in a partial solution of the primary problem concerned; namely, the degree of similarity of the blood proteins of some of the more closely related mammals, birds and reptiles. It seems very possible that a reliable serological relationship study of birds and of reptiles can be accomplished. Additional data may aid in clarifying the " taxonomic confusion " of birds.

Finally—a plea is made to the workers using the serologic method, especially the precipitin technique. It is desirable to obtain uniform and reliable results and one should use the methods that are best adapted to the particular problem. The earlier workers' contribution was and is invaluable but not necessarily the only one that is of use. A modification of the earlier methods is in order and only when one uses the best possible method for the particular study will the results be consistent and therefore biologically significant.

Summary

1. Antibodies were produced in rabbits by injecting very small quantities of serum proteins of mammals, birds and reptiles.

2. Antisera produced by injection of a minimum quantity of antigen were the most specific as determined by the "ring" method.

3. Occasionally the serum proteins of such closely related animals as ox and sheep or gray squirrel and red squirrel could be distinguished.

4. Antiserum dilutions as low as 1:3 at times eliminated the heterologous reaction.

5. The "ring" appeared in the homologous and very closely related protein solutions earlier than in the more distantly related ones when incubated at temperatures of 37° ± 1° C.

6. It was not possible to distinguish between the buffalo and ox serum proteins or between the goat and sheep serum proteins.

7. Serological relationship studies of birds and reptiles seem feasible.

8. The procedures have not been entirely new but the necessity for uniformity and standardization of methods has been emphasized.

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EXPERIMENTS ON LIGIA IN BERMUDA

VI. Reactions to Common Cations

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Five preceding papers of this series (Barnes, 1932, 1934, 1935, 1936, 1938) have described the effect of salts and other factors on the littoral isopod *Ligia baudiniana*. The present report deals with the negative reaction of *Ligia* to filter paper moistened with salt solutions and the survival in sea water containing a higher percentage of K, Ca, or Na.

Reaction to Filter Paper Moistened with Salt Solutions

Large filter papers (diameter 25 cm.) were cut in two, each half saturated with a different solution and placed in a covered flat dish in a photographic dark room having a light of 61.9 foot candles directly above the center of the dish. Five isopods (freshly collected) were released in the dish and their distribution on the two halves was observed at five to fifteen-minute intervals for a period of one to two hours. The dish was rotated 90° after each observation to eliminate any unsuspected source of orientation. With distilled water on both sides the distribution was approximately equal.

It is known (Barnes, 1938) that the isopods tend to collect on the distilled water side when the other half of the paper is moistened with sea water and the present experiments were designed to test the salts separately. The aversion for sea water is shown by specimens previously kept in air on seaweed moistened with sea water (Barnes, 1938) and by specimens washed rapidly in distilled water (Barnes, 1935), but it was found that prolonged immersion in distilled water destroys this reaction. Thus in the present experiments of 168 specimens previously immersed separately in 100 cc. of distilled water for an average of half an hour, 82 collected on the sea water side and 86 on the distilled water paper.

For $\frac{5}{8}$ M NaCl *vs.* distilled water, the ratio is the same as for sea water *vs.* distilled water, and in both cases the negative reaction no longer occurs at 50 per cent dilution (Table I). The aversion for KCl paper

was still evident in a dilution of 10 per cent (see Table I). The isopods showed a positive reaction to $CaCl_2$ paper compared with distilled water paper and an "all-or-none" reaction to $CaCl_2$ occurred when the other half contained NaCl. An even distribution occurred between $CaCl_2$ and LiCl paper and the negative reaction to LiCl compared with distilled water was less than that to the other salts.

Survival in Modified Sea Water

The toxic action of Na, Ca and K was studied by increasing the proportion of each salt in sea water. Specimens were tested separately in 100 cc. of mixtures of sea water and isotonic salt solution (Table II).

TABLE I

Reaction of Ligia to filter paper saturated with salt solutions.
(The animals were tested in groups of five.)

Treatment of each half of paper	Total number of isopods on each half	Ratio
5/8 M NaCl vs. distilled water	71 : 127	1 : 1.78
75 per cent 5/8 M NaCl vs. distilled water	85 : 120	1 : 1.41
50 per cent 5/8 M NaCl vs. distilled water	73 : 77	1 : 1.05
25 per cent 5/8 M NaCl vs. distilled water	83 : 72	1 : 0.86
5/8 M NaCl vs. $\frac{3.5}{8}$ M $CaCl_2$	1 : 63	1 : 63.00
100 per cent 5/8 M KCl vs. distilled water	37 : 147	1 : 3.97
75 per cent 5/8 M KCl vs. distilled water	26 : 82	1 : 3.15
50 per cent 5/8 M KCl vs. distilled water	31 : 81	1 : 2.61
25 per cent 5/8 M KCl vs. distilled water	56 : 82	1 : 1.46
10 per cent 5/8 M KCl vs. distilled water	61 : 99	1 : 1.62
5 per cent 5/8 M KCl vs. distilled water	96 : 104	1 : 1.08
$\frac{3.5}{8}$ M $CaCl_2$ vs. distilled water	105 : 55	1 : 0.52
$\frac{3.5}{8}$ M $CaCl_2$ vs. 5/8 M LiCl	22 : 22	1 : 1.00
5/8 M LiCl vs. distilled water	49 : 63	1 : 1.28

The isopods showed a high tolerance for sea water containing excessive sodium or calcium, but increasing the KCl content is rapidly fatal. The toxicity of Na is a smooth function of the concentration. The Ca curve is erratic and the K curve falls abruptly for sea water mixtures containing over $\frac{1}{16}$ M KCl (see Fig. 2).

Survival in Oxygenated Solutions

The wide variation in survival times in a given salt solution suggests

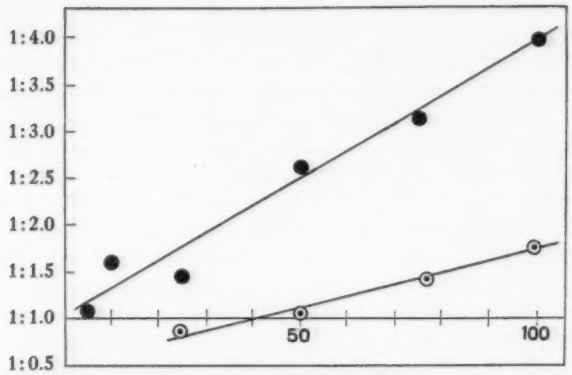

FIG. 1. Negative reaction to filter paper containing salt solutions. Ordinates: ratio of the number of isopods on the salt solution side to the number on the distilled water side. Abscissae: percentage concentration in cc. of 5/8 M salt. Solid circles: KCl. Open circles: NaCl (see Table I).

that other factors besides the direct chemical effect of the ions may be involved. To eliminate effects of asphyxiation oxygen was bubbled

TABLE II

Survival of Ligia in modified sea water

Parts of salt solution in 100 cc. of modified sea water	Average survival	Maximum survival	Number of specimens
	hours	*hours*	
9 per cent 5/8 M NaCl	69.8±15.2	136	9
50 per cent 5/8 M NaCl	59.4±4.5	168	39
71.4 per cent 5/8 M NaCl	30.9±3.5	79	20
80 per cent 5/8 M NaCl	20.7±0.8	24	37
90 per cent 5/8 M NaCl	8.0±0.03	11	43
10 per cent 5/8 M KCl	44.7±5.6	112	19
11 per cent 5/8 M KCl	36.5±5.5	48	8
20 per cent 5/8 M KCl	10.2±0.7	17	19
30 per cent 5/8 M KCl	3.7±0.4	6	8
40 per cent 5/8 M KCl	5±0	5	5
50 per cent 5/8 M KCl	0.7±.08	1	10
30 per cent $\frac{3.5}{8}$ M $CaCl_2$	68.5±10.8	264	25
50 per cent $\frac{3.5}{8}$ M $CaCl_2$	34.2±5.7	242	24
70 per cent $\frac{3.5}{8}$ M $CaCl_2$	39.1±6.7	254	19
90 per cent $\frac{3.5}{8}$ M $CaCl_2$	38.8±2.9	62	17

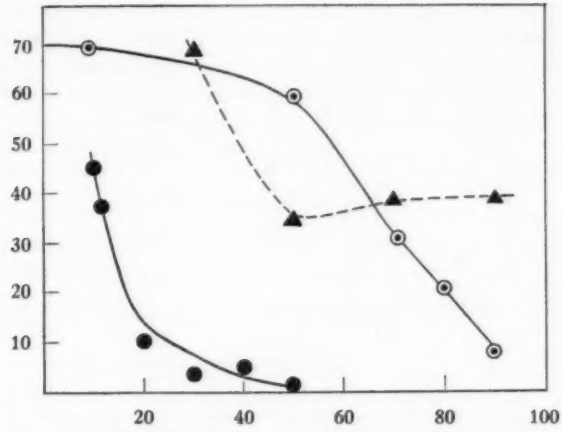

FIG. 2. Survival times of isopods in sea water in which the concentration of one ion is increased. Ordinates: average survival in hours. Abscissae: concentration of 5/8 M KCl and NaCl and of 3.5/8 M $CaCl_2$ in cc. per 100 cc. of modified sea water. Solid circles: KCl; open circles: NaCl; triangles: $CaCl_2$.

through the 100 cc. of salt solution in several cases, but did not affect the survival times in distilled water, $\frac{5}{8}$ M NaCl or $\frac{5}{8}$ M KCl (Table III).

TABLE III

Survival in oxygenated solutions

Medium	Average survival	Maximum survival	Number of specimens
	hours	*hours*	
Distilled water	4.0±0.2	6	8
5/8 M NaCl	8.7±0.6	11	4
5/8 M KCl	1.6±0.1	3	7

Discussion

It is of interest to note that the aversion for sea water is similar to aversion for NaCl and in both cases the negative reaction ceases at half dilution. The quantity of KCl in sea water is below the threshold for the negative reaction to this salt, while $CaCl_2$ has no repellent action when compared with distilled water.

It was suggested (Barnes, 1938) that the salts on the paper stimulate the isopods to greater movement which compels them to collect on the salt-free side. The pronounced aversion for KCl and positive reac-

tion to Ca support this hypothesis. The rather precise relation between the concentration of NaCl and KCl and the magnitude of the response may also be cited. It is unfortunate that our quantitative knowledge of chemical stimulation is very meager compared with the extensive studies of stimulation by light and gravity. The ratio of effect of K *vs.* distilled water to Na *vs.* distilled water is 2.2, somewhat greater than the ratio of ionic mobilities, i.e., 1.5. Hopkins (1932) has shown the importance of ionic mobility in stimulation by salts. The reaction of the animals when each side of the substratum is treated with a different salt is not an additive effect of the reactions to each salt compared with distilled water paper. Thus the ratio for $CaCl_2$ *vs.* distilled water is 1 : 0.52 and NaCl *vs.* water is 1 : 1.78 but the ratio for $CaCl_2$ *vs.* NaCl is as high as 63.0 : 1. This striking difference probably results from the combined action of the inhibiting Ca and stimulating Na on the leg movements. The differences cannot be explained by toxicity alone. Thus Li, the most toxic of the ions, gives a 1 : 1 ratio with Ca.

In the case of sea water *vs.* distilled water the salt requirements of the animals must also be considered. Thus if depleted of salt by a half hour's immersion in distilled water the negative reaction to sea water disappears. The rôle of the flushing mechanism for the gills by which water rises between the last pair of legs is not known. Several animals were observed with the legs in position for the capillary conduit, but there was not sufficient solution on the filter paper to flush the gills.

The specific effect of Na, Ca, and K is indicated in the toxicity curves for sea water containing increasing concentrations of each ion. The smooth relation between concentration and toxicity for Na suggests a gradual modification of a normal process in the animal. The very steep KCl curve indicates severe toxic action and the lack of correlation with concentration in the case of calcium is characteristic of a surface reaction. It was shown (Barnes, 1938) that the protective action of Ca in hypotonic sea water also resembles a surface reaction.

The experiments in which oxygen was introduced into the solutions help to show that the reported survivals of immersed animals are true salt effects. This is especially important in the case of K which paralyzes all gill movement. It is probable that oxygen would increase the survival in less toxic solutions in which the animals live for several days.

Summary

1. The negative reaction of *Ligia* to filter paper moistened with $\frac{5}{8}$ M NaCl is similar to the negative reaction to sea water. In both cases dilution by 50 per cent destroys the effect.

2. The aversion for filter paper containing KCl and NaCl is approximately a rectilinear function of the concentration.

3. The results can be explained by the assumption that the ions stimulate the legs and the greater activity results in a distribution on the salt-free side of the substratum.

4. When presented with a "choice" between filter paper containing $CaCl_2$ and NaCl, there is an "all-or-none" aversion for the NaCl.

5. The survival of *Ligia* in sea water containing increasing concentrations of Na, K and Ca is described.

6. The survival in distilled water or in solutions of single salts is not affected by oxygenation.

The writer is indebted to Dr. J. F. G. Wheeler for many courtesies.

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